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Original Article

Fluoxetine and diclofenac interaction on food intake in Goldfish, *Carassius auratus*

Mohammad Navid Forsatkar[1], Maryam Hedayatirad[1], Kazem Kookaram[1], Mohammad Ali Nematollahi*[1], Wen-Bin Huang[2]

[1]Faculty of Natural Resources, Department of Fisheries, University of Tehran, Karaj, Iran.
[2]Department of Natural Resources and Environmental Studies, National Dong Hwa University, Hualien, Taiwan.

Abstract: This study was carried out to investigate the interaction of simultaneous use of fluoxetine (Flx), a selective serotonin reuptake inhibitor, and diclofenac (Dcf), a non-steroidal anti-inflammatory drug, on food intake in goldfish, *Carassius auratus*. Treatments with different dosage of Flx including control, 0, 1, and 10 µg/g body weight (BW) were injected in the fish with mean weight of 30.16 ± 8.57 g every other day in total of 5 times. Then fish were exposed to 3 different levels of Dcf including 0, 10, and 100 mg/l for 5 days. Injection of fluoxetine significantly decreased food intake and consequently body weight. After 5 days exposure to Dcf, the amount of food intake in the Dcf receiving treatments of 1 mg/l and 10 mg/l was significantly larger than that of 0 mg/l Dcf receiving treatment in both the Flx dosage groups of 1 µg/g BW and 10 µg/g BW. Our results indicated that Dcf inhibits behavioral change effects of Flx showing the complex effects of pharmaceuticals on fish.

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Introduction

Study on the antagonistic relationship between nonsteroidal anti-inflammatory drugs (NSAIDs) and antidepressant pharmaceuticals indicated that the NSAID significantly reduced antidepressant-like effects of SSRIs but had less effect on other types of drugs, and concluded that reduced use of NSAIDs in patients with depressive disorders can increase the efficiency of treatment with SSRIs (Warner-Schmidt et al., 2011). In addition, simultaneous use of NSAIDs would increase risk of gastrointestinal bleeding associated with SSRIs intake. Furthermore, the antinociceptive action of NSAIDs is modulated by the serotonergic system (Miranda et al., 2003). Therefore, more study is needed to assess the complex relationship between NSAIDs and SSRIs and their interactions.

Flx is one of the most common SSRI antidepressants (Mennigen et al., 2010), which is metabolized in the liver by the cytochrome P450 enzyme (especially CYP2D6) (Hiemke and Hartter, 2000). Levels of ingested drug are excreted in urine as two forms of parent compound and its main metabolite, nor-fluoxetine (de Vane, 2000). These compounds pass through biological treatment in wastewater treatment plants (Gaworecki and Klaine, 2008) and the reported environmental concentration range of Flx is 12 ng/l to 540 ng/l (Calisto and Esteves, 2009). Flx is a lipophilic drug indicating its ability to accumulate in tissues (Hiemke and Härtter, 2000). The brain and the liver are the main sites for accumulation of Flx in non-target organisms, such as fish (Brooks et al. 2005), that have similar actions in both fish and mammalian brain (Mennigen et al., 2009).

Dcf has special pharmacological properties. The activation of cyclooxygenases (COX) is inhibited by Dcf, leading to prevent prostaglandin synthesis and subsequently pain is abated (Miranda et al., 2003). Consumption of 85.80 tons of Dcf in Germany only in 2001 (Huschek et al., 2004) represents the environmental occurrence of this drug in aquatic

* Corresponding author: Mohammad Ali Nematollahi
E-mail address: malahi@ut.ac.ir

systems. Concentration ranges of Dcf in German rivers has been reported at 0.15-1.2 µg/l (Ternes, 1998). Like other drugs, Dcf has adverse side effects which relate to inhibition of prostaglandin synthesis (Hoeger et al., 2005). Pathogenic alterations in gill and kidney of rainbow trout has been reported after Dcf exposure (Schwaiger et al., 2004). Twenty-one days of Dcf exposure to brown trout (*Salmo trutta*) resulted in the breakdown of gill lamella and trunk kidney; lysozyme activity increased significantly after 21 days in a 5 µg/l exposure group; however Haematocrit value was not significantly different (Hoeger et al., 2005).

It is well known that alteration in behavior and physiological functions are related to occurrence of disrupting chemicals. In mammals, physiological mechanisms of food intake in relation to changes in serotonin levels have been well investigated (Leibowitz, 1985). It has been shown that any process associated with increases in the serotonergic system may induce satiety and reduce appetite (Simansky et al., 1992). Flx, which inhibits reuptake of serotonin from synaptic junctions, promotes the extracellular serotonin levels within the brain (Hemeryck and Belpaire, 2002). In fish, de Pedro et al. (1998) reported that intracerebroventricular injection of serotonin in goldfish, *Carassius auratus*, significantly reduced food intake 2 hours post injection, however, intraperitoneal administration of serotonin had low effect. They concluded that serotonin acts as a potential factor to control appetite in goldfish.

There are many studies on the effects of sub-lethal concentrations of pharmaceutical agents alone in aquatic organisms, especially fish. However, influence of multiple drugs together and interactions between them on fish physiology and behavior has been given less attention. Given the complexity of pharmaceutical contaminants in the environment, special attention to this part of ecotoxicological knowledge is needed. Therefore, the aim of the present study was to investigate the effects of Flx injection and Dcf exposure on food intake and weight gain of goldfish. This species is an excellent model to evaluate food intake (Mennigen et al., 2009), and has been the subject of many growth studies.

Material and methods

Animals: One hundred and fifty goldfish, *C. auratus*, with an average weight of 30.16 ± 8.57 g were purchased from a commercial fish farm in Rasht, Iran. They were divided equally and kept in five 100-liter fiberglass tanks with constant aeration, water temperature of 21-23 °C, and a photoperiod of 12L: 12D. Half of the water in the tanks was replaced with dechlorinated tap water every 2 days from the beginning of a 3-week acclimation period until the beginning of the Dcf exposures. Fish were fed daily at 11:00-11:30 with commercial pellets at a rate of 2% of body weight (BW). This amount of food is enough for optimal growth in goldfish (Volkoff et al., 1999).

Chemicals and procedures: There were 2 experiments in this study, including experiment-I, Flx injection, and experiment-II, connected Dcf exposure. In experiment-I, an 8 mg/ml Flx solution was prepared with 8 mg of fluoxetine HCl dissolved in 1 ml of 0.9% physiological saline. Concentration of the Flx solution was diluted with saline for intraperitoneal injections. Nine fish were randomly selected from the five 100-liter fiberglass tanks, weighed, and put into a 25-liter experimental tank (9 individuals per tank). There were 12 experimental tanks that were randomly divided into 4 Flx injection dosage treatments (3 replicate tanks per treatment), including no injection (control, Flx C), 0.9% saline only (Flx 0), 1 µg/g BW (Flx 1), and 10 µg/g BW (Flx 10). Fish were anesthetized with extract of clove powder (20 mg/l) and injected intraperitoneally with the Flx dosage of its treatment group every other day in the 9 days of the experiment-I. A total of 5 injections were performed. A food intake test was done 15 minutes after each injection for each tank.

In experiment-II, a 400 mg/ml Dcf solution was prepared with 2000 mg of diclofenac sodium dissolved in 5 ml of ethanol for Dcf exposures. Three Dcf exposure concentration treatments, no Dcf

Table 1. Two-way ANOVA table for the food intake affected by the Flx injection treatments crossed with the Dcf exposure treatments. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Source of variation	SS	df	MS	F	P
Flx injection	9.846	3	3.282	21.648	0.000***
Dcf exposure	1.343	2	0.671	4.429	0.023*
Interaction	6.171	6	1.028	6.784	0.000***
Error	3.639	24	0.152		
Total	20.998	35			
Source of variation	SS	Df	MS	F	P
Dcf exposure effects in the Flx group of					
Control	0.815	2	0.408	3.051	0.122
0 μg/g Flx	0.634	2	0.317	2.703	0.146
1 μg/g Flx	2.366	2	1.183	5.525	0.044*
10 μg/g Flx	3.699	2	1.849	13.077	0.006**
Flx injection effects in the Dcf group of					
0 mg/l Dcf	11.706	3	3.902	19.937	0.000***
1 mg/l Dcf	3.502	3	1.167	6.100	0.018*
10 mg/l Dcf	0.808	3	0.269	3.978	0.053

addition (Dcf 0), 1 mg/l Dcf (Dcf 1), and 10 mg/l Dcf (Dcf 10), were used for 5-day Dcf exposures in experiment-II which began continually on the 4th day after accomplishment of experiment-I. The 3 tanks in each Flx dosage injection treatment of experiment-I were randomly assigned to the 3 Dcf exposure concentration treatments. In each tank, the 9 fish were randomly separated into 3 sub-tanks (3 individuals per sub-tanks) which formed 3 replicates per Dcf exposure treatment. Therefore, 3 Dcf concentration exposure treatments crossed with 4 Flx dosage injection treatments with 3 replicates (sub-tanks) were tested. After 5 days of Dcf exposures, the food intake tests, 36 in total, were done.

Execution of the food intake test followed Mennigen et al. (2009) and de Pedro et al. (1998). Fish were not fed for one day before the Flx injections as well as one day before the Dcf exposures. Fifteen minutes after the injection in the first 9 days as well as the day after the 5-day Dcf exposures, fish were given excess food with commercial pellets at a rate of 4% of BW. One hour after feeding, residual food was slowly siphoned out and dried in an oven at 60°C for 2 hours, then weighed. Food intake (FI) was calculated with the formula: $FI = W_i$ (initial dry food weight) – [Wf (residual dry food weight) × F (correction factor)], which F, determined by residual ratio of the dry weights of commercial pellets kept in water alone for 1 hour, was 0.88 ± 0.02 (n = 3).

To calculate weight gain, fish weight was measured 3 times: (1) before Flx injections, after Flx injections, and at the end of Dcf exposure. Until end of injections, 9 fish in each tank, were weighed individually and weight gain calculated by subtracting the initial weight from the final. For weight gain calculation during Dcf exposure, 9 fish in each tank were divided into 3 groups of 3 individuals as one replicate and weighed. At the end of exposure, those 3 fish were weighed again and weight gain calculated as before.

Statistical analysis: Two-way and one-way ANOVAs followed by the Duncan's multiple range tests ($P<0.05$) were used to determine significance

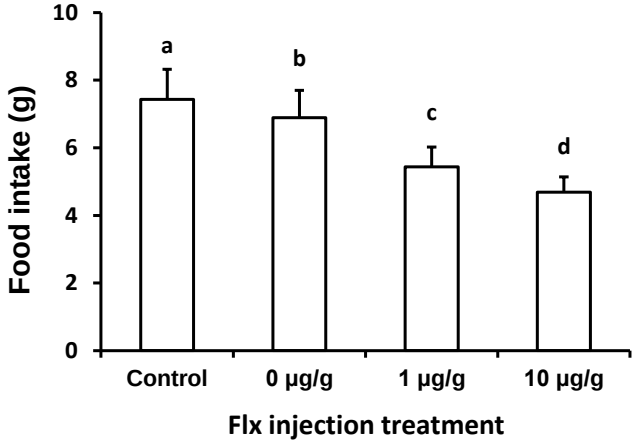

Figure 1. Means ± standard deviations of the food intake (g per fish per day) of goldfish (*Carassius auratus*) in the 4 Flx injection treatments. n = 15 (3 replicate tanks with 5 injection times); Letters that differ indicate significance differences at $P<0.05$.

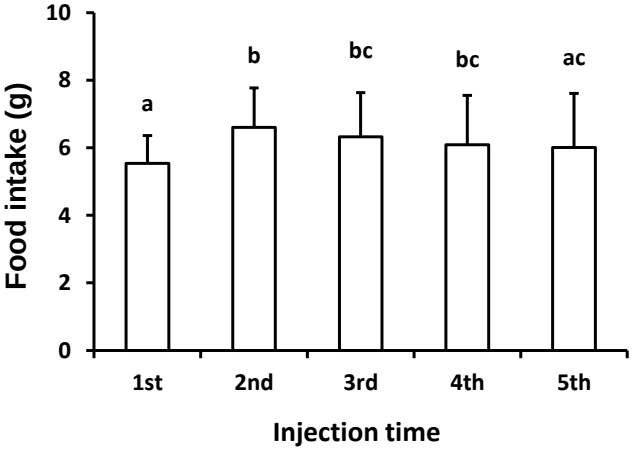

Figure 2. Means ± standard deviations of the food intake (g per fish per day) of goldfish (*Carassius auratus*) in the 5 injection times. n = 12 (3 replicate tanks with 4 Flx injection treatments); Letters that differ indicate significance differences at $P<0.05$.

differences of the food intake among the 4 Flx dosage treatments crossed with the 5 injection times and the weight gain among the 4 Flx dosage treatments in experiment-I, respectively. The two-way ANOVA was also used to determine significance differences of the food intake and the weight gain among the 3 Dcf exposure treatments crossed with the 4 Flx dosage treatments in experiment-II. Statistics were performed using SPSS 19.0 software.

Results

In experiment-I of the Flx treatment, results of the two-way ANOVA showed that the food intake was

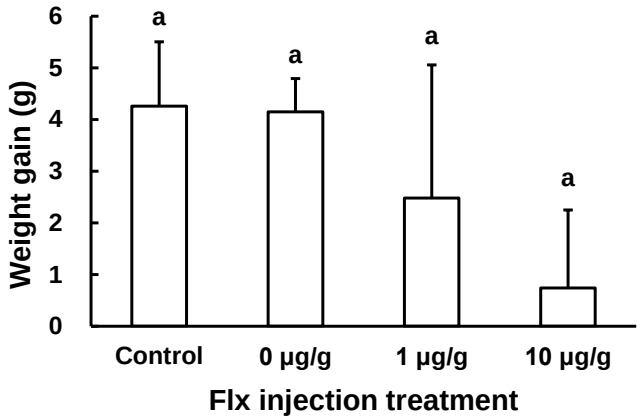

Figure 3. Means ± standard deviations of the weight gain (g per fish per day) of goldfish (*Carassius auratus*) after the 5 time injections in the 4 Flx injection treatments. n = 3; Letters that differ indicate significance differences at $P<0.05$.

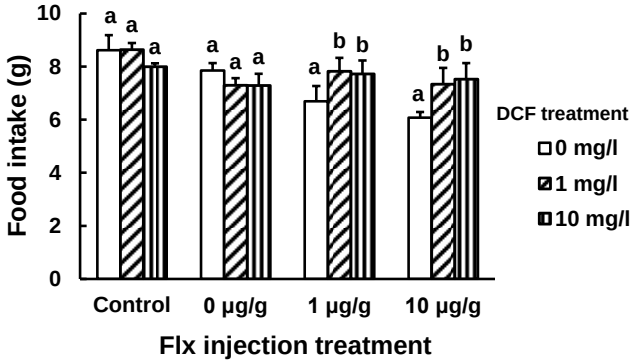

Figure 4. Means ± standard deviations of the food intake (g per fish per day) of goldfish (*Carassius auratus*) in the 4 Flx injection treatments crossed with the 3 Dcf exposure treatments. n = 3; Letters that differ indicate significance differences at $P<0.05$.

significantly affected by both the main factors, the Flx dosage ($F_{3,40}$=66.820, P=0.000) and the injection time ($F_{4,40}$ =5.222, P=0.002) but not by the interaction ($F_{12,40}$=1.342, P=0.234). The food intake was significantly the lowest in the Flx 10 treatment, followed by the Flx 1 treatment and the Flx 0 treatment, and the largest in the Flx C treatment (Fig. 1). The food intake was the lowest after the first injection, significantly increased to the largest after the second injection, and decreased moderately from the third injection to the fifth injection (Fig. 2). After the 5 time injections, a dramatic standard deviation of the weight gain in the Flx 1 treatment was found while means of the weight gains were insignificantly different among the 4 Flx treatments ($F_{3,8}$=3.041, P =0.093) even though the means in the Flx 10 and Flx

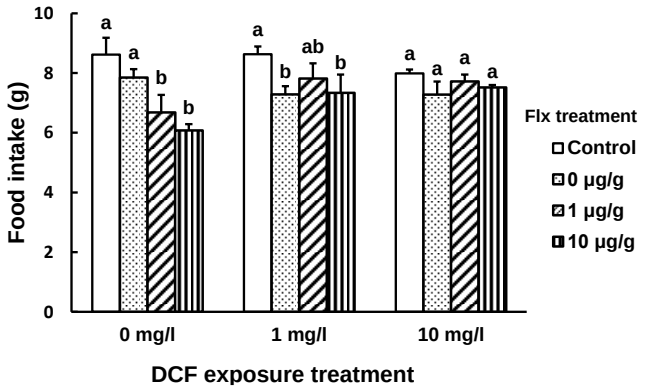

Figure 5. Means ± standard deviations of the food intake (g per fish per day) of goldfish (*Carassius auratus*) in the 3 Dcf exposure treatments crossed with the 4 Flx injection treatments. n = 3; Letters that differ indicate significance differences at $P<0.05$.

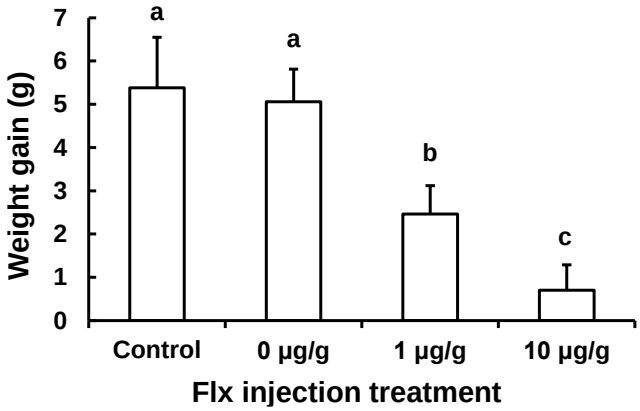

Figure 6. Means ± standard deviations of the weight gain (g per fish per day) of goldfish (*Carassius auratus*) in the 4 Flx injection treatments. n = 9 (3 replicate tanks with 3 Dcf exposure treatments); Letters that differ indicate significance differences at $P<0.05$.

1 treatments were smaller than those in the Flx 0 and Flx C treatments (Fig. 3).

In experiment-II of the Flx injection crossed with the Dcf exposure, results of the two- way ANOVA showed that the food intake was significantly affected by both the main factors, the Flx dosage ($F_{3,24}$=21.648, P=0.000) and the Dcf exposure ($F_{2,24}$=4.429, P=0.023), and also by the interaction ($F_{6,24}$=6.784, P=0.000). The effect of the Dcf exposure treatments on the food intake was not significant in both the Flx C group ($F_{2,24}$=3.051, P= 0.122) and the Flx 0 group ($F_{2,24}$=2.703, P=0.146) but was significant in both the Flx 1 group ($F_{2,24}$= 5.525, P=0.044) and the Flx 10 group ($F_{2,24}$=13.077, P=0.006) (Table 1). In both the Flx 1 and Flx 10 groups, the food intakes in the Dcf 1 and Dcf 10 treatments were significantly larger than that those in the Dcf 0 treatment (Fig. 4, right 2 panels).

The effect of the Flx injection treatments on the food intake was not significant in the Dcf 10 group ($F_{3,24}$=3.978, P=0.053) but was significant in both the Dcf 0 group ($F_{3,24}$=29.937, P=0.000) and the Dcf 1 group ($F_{3,24}$=6.100, P=0.018) (Table 1). In the Dcf 0 group, the food intake was significantly lower in the Flx 1 and 10 treatments than those in the Flx 0 and Flx C treatments (Fig. 5, left panel). In the Dcf 1 group, the food intake was the lowest in the Flx 0 and Flx 10 treatments, followed by the Flx 1 treatment, and the largest in the Flx C treatment (Fig. 5, middle panel).

The Results of two-way ANOVA in experiment-II showed that the weight gain was significantly affected only by the Flx injection treatment ($F_{3,24}$=52.886, P=0.000) but not by the Dcf exposure treatment ($F_{2,24}$=0.064, P=0.938) and the interaction ($F_{6,24}$=0.265, P=0.948). The weight gain was significantly the lowest in the Flx 10 treatment, followed by the Flx 1 treatment, and the largest in the Flx 0 and control treatments (Fig. 6).

Discussion

There are many parameters regulating food intake in fish, including metabolic, neuro-physiological, and hormonal mechanisms. Also, environmental conditions indirectly affect the appetite. In mammalian species, Lam and Heisler (2007) showed that the serotonergic pathway is involved in appetite regulation and energy homeostasis. Some evidence in humans suggests an inhibitory role of Flx on appetite and weight gain (Halford et al., 2007). In our study, the amount of food intake significantly decreased after Flx injection treatments of 1 µg/g BW and 10 µg/g BW. This indicates that the Flx had a negative effect on appetite of the goldfish. After the second injection, food intake of all treatment groups increased in comparison to the first injection. This increase may be related to manipulation stress in fish. The first food intake test performed immediately after the acclimation period where the fish were divided from 100-liter tanks to smaller 50-liter ones; while in the second test, fish were adapted

to the new situation.

The results show that Flx affects weight gain of goldfish, although it was not significant in our study. The effect of Flx on human nutrition is contradictory. Some cases reported a repressive action (Pijl et al., 1991); for this, higher doses of Flx are needed i.e., several times greater than the dose used for treatment of depression. Fogelson (1991) showed that Flx has no effect or even additive action on weight gain. In this study, after five injections of Flx, the lowest weight gain was observed in Flx 10 group. This is because food intake in this group was less than other treatment groups. With lower food consumption, fish remained under starvation condition and did not have enough energy to devote to growth and weight gain. Also, the potential impacts of Flx on appetite parameters such as increased secretion of Crf (de Pedro et al., 1998) and reducing NPY (Lopez-Patino et al., 1999), causes negative weight gain after administration of Flx. Similar results are observed in rats in which Flx administration after 44 days prevented weight gain (Cantor et al., 1999). Mennigen et al. (2010) reported that Flx exposure caused 7-fold reduction in goldfish weight after a 28 day experiment. Study on fat mice showed the main effect of Flx on lipid tissues and protein content has slight reduction (Gutierrez et al., 2002). Therefore, only 9 days duration in our study might be too short to bring out the entire effect of the Flx treatments, which come out insignificantly in the results. In addition, the small sample size (n=3) and high variance among the 3 replicates (dramatic standard deviation) could be one of the other reasons why the effect of the Flx was masked in our study.

After the Dcf exposure, the food intake was significantly larger in the Dcf exposure treatments of 1 mg/l and 10 mg/l than that in the 0 mg/l Dcf exposure treatment in both the Flx dosage groups of 1 μg/g BW and 10 μg/g BW. These results show the inhibitory role of Dcf on the Flx side effect of appetite reduction. de La Garza and Asnis (2003) reported that Dcf sodium reduces the serotonin turnover in the prefrontal cortex of rat brains. Concentration of serotonin in goldfish tissue was not measured in our study, but it is known the serotonergic pathways are affected by Flx (Mennigen et al., 2010) and/or NSAIDs (Warner-Schmidt et al., 2011). There is a central anorectic action of serotonin in teleost fish (de Pedro et al., 1998), as a result, Flx can reduce food intake by increasing serotonin levels and promoting the amount of food intake is related to the antagonistic relationship of Dcf exposure on Flx.

After 5 days Dcf exposure, insignificant results of the Dcf effects on the weight gain were found in this study, although the food intake recovered due to the Dcf exposure effects in the Flx dosage groups of 1 μg/g BW and 10 μg/g BW. Cleuvers (2003) indicated that models of toxicity mixture between Flx and Dcf is synergistic because the combined effects of these two compounds are more than the sum of the effects of each alone. Therefore, only 5 days Dcf exposure duration in our study might has been too short for the entire effect of the Dcf treatments to emerge. This could be one of the main reasons why the effect of the Dcf was insignificantly in the results.

In conclusion, Flx injection can significantly affect food intake and weight gain in goldfish. In the relationship between Flx, a potential augmentor of extracellular serotonin levels, and Dcf, a cyclooxygenase (COX) enzyme inhibitor, it was found that Dcf exposure regulated Flx impacts on appetite and growth conditions. Molecular and physiological studies are needed to further understand the effects of multiple compounds interaction on aquatic organisms.

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