

# Fish on drugs

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An experimental study of crucian carp exposed to fluoxetine and a  
natural predator

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# Abstract

Organisms in the aquatic environment face the challenge of exposure to environmental pollution by a multitude of pharmaceuticals. However, the potential for disruptive effects in non-target aquatic organism remains poorly understood. Fluoxetine is one of the most commonly prescribed psychoactive drugs and it has been shown to bioaccumulate in fish, and potentially affects a broad suite of important traits. Changes in fish morphology, behaviour and habitat use can have consequences at ecosystem level, since cascading effects can impact organisms of several trophic levels. Hence, a deeper understanding of potential responses of non-target organisms to fluoxetine exposure under various conditions is much needed. Here, I investigate single and combined effects of short-term exposure (c.a. 18 days) to two stressors; the anti-depressant fluoxetine and predation risk on three important fitness related traits (boldness, sociability and body weight) in the crucian carp (*Carassius carassius*), a common freshwater fish. My results show that exposure to fluoxetine at  $100 \mu\text{g L}^{-1}$  significantly decreased propensity to take risks (boldness) in crucian carp, but I found no effects of fluoxetine exposure on sociability or body weight. Furthermore, no effects of exposure to a predator (pike) or any interactions between fluoxetine and predator exposure on any of the traits measured were detected. My data shows that a common anti-depressant drug frequently found in nature can alter important fitness related traits in a species known for its resilience to abiotic disturbances, and hence I stress the need for further studies. For society to recognise and fully understand negative impacts of pharmaceuticals on fresh water ecosystems, I for future studies suggest an ecosystem approach, where several species, stressors, key behavioural and morphological traits under varying environmental conditions are considered.



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# Introduction

Human societies critically depend on healthy freshwater ecosystems, and yet big parts of the world's surface waters face a broad suite of anthropogenic stressors associated with human activities. Environmental pollution of aquatic environments is threatening the water quality of many surface waters, and therefore puts numerous species and key ecosystems at severe risk. A great variety of chemicals end up in nature via for example wastewater treatment plants (WWTPs). The mixture of pollutants in recipients is troublesome since their synergistic effects are difficult to understand and predict, especially when combined with the concept of trophic cascades (Carpenter et al. 1985). If different organisms respond differently to pollutants, relationships within and between species can be altered via changes in individuals' ability to compete and avoid predation, with major consequences for ecosystems functioning and resilience. It is thus important that the consequences of release of different chemical substances are studied, in order to enable adequate risk assessments so that society can take necessary preventive measures to combat and reduce the negative impacts of environmental pollution.

Recently, pharmaceuticals and their potential to affect non-target biota have received more attention, since many organisms have similar physiology as humans and thus are suspected to be sensitive to drugs originally designed to target humans exclusively. One example of this is the presence of pharmaceuticals in the aquatic environment targeting the serotonergic system that many diverse organisms share (Winberg & Nilsson 1993; Lillesaar 2011). Serotonin, a key neurotransmitter in the central nervous system, is involved in for example social locomotion, aggression and fear expression in fish (Lillesaar 2011) and thus important for fitness and survival. Several different selective serotonin reuptake inhibitors (SSRIs) have been reported from recipients of WWTP effluents (e.g. Vasskog et al. 2006, Schultz et al. 2010). They are commonly used to treat afflictions linked to serotonin levels like depression, anxiety, obsessive-compulsive disorders connected to Tourette's syndrome and eating disorders (Kreke & Dietrich 2008). These compounds are designed to attach to serotonin reuptake transporter enzymes in synaptic clefts, so that serotonin fail to attach. This results in serotonin remaining free in the cleft, stimulating further signals in the post-synaptic cell, instead of being reuptaken in the pre-synaptic cell (e.g. Kreke & Dietrich 2008). Thereby, SSRIs function by modifying levels of serotonin. Fluoxetine is one of the most prescribed SSRIs (Grohol 2013) and commonly found outside WWTPs in concentrations below  $1 \mu\text{g L}^{-1}$  (Mennigen et al. 2011). It is one of the more stable SSRIs with a half-life time of 1-4 days (Heimke & Härtter 2000; Kreke & Dietrich 2008). Its primary metabolite norfluoxetine has an even longer half-life time of 7-15 days (Heimke & Härtter 2000; Kreke & Dietrich 2008), and similar effects on the serotonergic system as the parent compound (Kreke & Dietrich 2008). With more or less constant addition to recipients via wastewater due to poor removal in WWTPs (Hedgspeth et al. 2012), organisms can be chronically exposed to both fluoxetine and norfluoxetine. Contemporary studies have shown that these substances can bioaccumulate in fish (Brooks et al. 2005, Schultz et al. 2010), organisms known to play a key role in the trophic dynamics of freshwaters (Carpenter et al. 1985). The potential for bioaccumulation raises concern, since exposure to SSRIs has been shown to alter various fitness traits, including behaviour, morphology, physiology and life history in several organism groups and thereby possibly disturb biological interactions (e.g. Bossus et al. 2014; Dzieweczynski & Hebert 2012; Fong & Ford 2014; Hedgspeth et al. 2014; Mennigen et al. 2011; Perreault et al. 2003; Weinberger II & Klaper 2014).

Although SSRIs have been shown to alter several organismal traits when studied separately, animals in nature typically have to cope with a suite of environmental stressors and threats. Hence, there is an urgent need to take a comprehensive approach and to understand the way different, simultaneously acting

stressors impact organisms. I am here interested in investigating not only the effect of fluoxetine itself, but also if there are synergistic effects when the drug is combined with other common stressors. As my model organism crucian carp (*Carassius carassius*) is extremely vulnerable to predation (e.g. Brönmark & Miner 1992; Brönmark et al. 1995; Holopainen et al. 1997a; Tonn et al. 1989), it is my aim to assess how the natural predator pike (*Esox lucius*) in combination with exposure to fluoxetine may alter important behaviours and physiological traits in crucian carp, further discussed below. As the species generally is considered to tolerate relatively high levels of abiotic disturbances like hypoxia and pollution (Holopainen et al. 1997b; Yi et al. 2007) any effects of fluoxetine would raise concern for impacts on more sensitive species.

To investigate single and combined effects of exposure to two stressors (fluoxetine and predation risk) on two key behavioural traits (boldness and sociability) and one physiological trait (body weight) in crucian carps, I exposed wild caught carps to three different concentrations of fluoxetine combined with absence or presence of pike. Boldness is commonly used to describe the personality of individuals in regard to their risk-taking behaviour (Budaev & Brown 2011). Increased boldness is thought to be a necessity in fish experiencing constant stress from predators – in order to maintain activities such as foraging and mating (Brown et al. 2005). Similarly, sociability has long been recognized as a behaviour that increases individual fitness in many fish species as predator avoidance and foraging is optimized (e.g. Morgan 1988). Body weight is also linked to fitness as a general measure of physiological status.

## Aim and hypotheses

The aim of this study is to investigate how two in nature occurring stressors; exposure to fluoxetine and risk of predation, separately and combined, alters three key traits in crucian carp; boldness, sociability and weight.

In accordance with the results of previous studies I hypothesised an effect of fluoxetine on carps, where exposed fish would be less bold (e.g. due to results showing decreased aggressiveness (Perreault et al. 2003) and locomotor activity (Winder et al. 2012)), less social (Xie et al. 2015) and lose more weight (Mennigen et al. 2010; Gaworecki & Klaine 2008) than unexposed fish. Similarly, I also hypothesised that crucian carps exposed to a predator would express increased boldness (Hulthén et al. 2014), increased sociability (Hoare et al. 2000) and gain more body weight (Hulthén et al. 2014) than carps held in an experimental environment absent of predators. Furthermore, as there is a possibility that fluoxetine may disrupt predator responses, I also hypothesized that there if that is the case, would be interactions between fluoxetine and predator exposure.

# Methods and materials

## Experimental animals

We used wild-caught crucian carp collected with dip nets in Lake Trollsjön, southern Sweden (55°50'15.3" N, 13°17'16.4" E), between the 20<sup>th</sup> and 22<sup>nd</sup> of April, 2015. We transported captured individuals (length 104 - 120 mm) to experimental facilities at Lund University where they were individually tagged by surgically implanting a TIRIS Passive Integrated Transponder (PIT) tag (Texas Instruments, RI-TRP-RRHP, Plano, Texas, USA, half duplex, 134 kHz, 23.1 mm long, 3.85 mm diameter, 0.6 g in air) into the coelomic cavity of the fish following Skov et al. (2005). These uniquely coded electronic tags allowed us to keep track of each individual during the entire experiment without altering external appearance. Thereafter, fish were allowed to acclimate for 4 weeks in a filtered and aerated 385-l tank. Food (pellets, frozen shrimp mix and gammarus) was supplied ad libitum during the acclimation period. Small pike (n=12) were collected in lake Krankesjön, southern Sweden and maintained individually for at least 8 weeks prior to the experiment.

## Behavioural assays

To investigate if fluoxetine and predation risk alters fish behaviour, both separately and combined, we quantified two key-behavioural traits (boldness and sociability) of individual carps before and after treatment exposure (13<sup>th</sup> of May – 8<sup>th</sup> of June and 10<sup>th</sup> of July – 15<sup>th</sup> of July, respectively). All behavioural trials were conducted in aged and aerated tap water of similar temperature as in the acclimation and exposure treatments tanks. To decrease environmental disturbance, a tarpaulin tent sheltered the experimental apparatus during all behavioural trials.

## Refuge emergence (boldness)

To obtain an index of individual boldness, we used an established refuge emergence protocol where the boldness score is defined as the latency to emerge from a refuge box (Brown et al. 2007). The experimental arena consisted of a circular PVC tank (volume 70 l), lined with ScotchLite luminous tape in order to facilitate behavioural analysis by providing a strong background contrast to the fish. Within the arena, we placed a refuge box (28×20×20 cm) made of grey PVC. Each trial started when one individual in the holding tank was haphazardly chosen and transferred to the refuge box. Each fish was given 5 min to acclimatize in the box, and when this time had elapsed, a vertically sliding trapdoor was slowly raised via a remote pulley system. We then observed refuge emergence behaviour via a video camera (Logitech C920) centrally mounted above the tank and linked to a monitor placed outside the tent. Observations were made with the criterion that the whole body of the fish had to be outside the box, and the time taken to emerge was recorded to the nearest 1 s. Each trial lasted for 10 min, and fish that had failed to emerge were given a ceiling value of 600 s.

## Tendency to shoal (sociability)

The experimental arena consisted of an aquarium (45 cm high × 45 cm wide × 125 cm long) divided lengthwise into three compartments (two small, 37 cm long, and a one large centre compartment, 49 cm long). Dividing walls were made from clear Plexiglas and sealed with silicone sealant to allow visual, but not physical or olfactory, interaction between the stimulus shoal and the focal individual. Opaque plastic covered the outer sides of the aquarium. For the shoal, we used a pool of 100 carps of similar size and from the same lake as the experimental animals. Each trial started when fifteen fish were drawn from this pool haphazardly and randomly allocated to one of the smaller compartments, while the other compartment was left empty. Next, a focal fish was introduced into the larger central compartment and allowed to acclimate for 10 min. A webcam positioned centrally above the arena and connected to computer remotely recorded the movements of the focal fish for 10 min. To obtain a quantitative measure of shoaling, the central compartment was divided with vertical marks every third cm and we calculated the proportion of time spent during 10 min by the focal fish within the zone 15 cm closest to the stimulus shoal.

## Experimental treatment

To assess how the presence of dissolved fluoxetine and the presence of a predator may affect fish behaviour we set up 24 aquaria (volume 165 l each). Each aquarium was divided into two compartments, a larger one (52×40 cm) to hold the crucian carp and a smaller compartment (42×40 cm) to hold pike in aquaria used for predator treatments. Perforated Plexiglas divided the compartments and three sides of each aquarium were externally covered with a black plastic film in order to prevent visual interactions between replicate aquaria. Fluoxetine HCl (CAS# 59333-67-4, Toronto Research Chemicals Inc.) stock solutions were prepared in ethanol (95%, Solveco, analytical grade) and stored at 4 °C. On day zero of the experiment, three different concentrations of fluoxetine was added to aquaria for chemical exposure treatments and single pike were allocated to the smaller compartment in aquaria used for the predator treatment. The experiment was initiated the day after spiking (day 1) when crucian carps were allocated to the larger compartment in all aquaria. Fish were randomly assigned to various fluoxetine and predator exposure treatments in a 2×3 design with the factors pike (presence/absence) and fluoxetine (three nominal concentrations of 0, 1 and 100 µg L<sup>-1</sup>, used in previous studies of fish behavioural changes by e.g. Weinberger II & Klaper (2014)). Each exposure treatment was replicated four times. Crucian carps were fed pellets, frozen gammarus and shrimp mix five times weekly and the food delivery rate corresponded to 5% of the weight of the carps in each replicate tank (nominally six individuals per tank). Pike were fed two crucian carps every week. After 14 days of exposure, approximately 75% of the aquaria water was replaced (day 15). In connection to this, aquaria were re-spiked with fluoxetine to initial levels. After treatment exposure, crucian carps were re-assayed for boldness and sociability (day 18 – 23). Post-treatment trials were conducted in sets of twelve (boldness) and six (sociability) replicate tanks daily to ensure comparability of hunger levels, time of day and so on. Environmental parameters as pH, oxygen levels, conductivity and temperature were continuously monitored.

## Data treatment and statistical analysis

The effects of fluoxetine and predator exposure on fish behaviour (boldness, sociability) and body weight were tested with nested ANOVAs. In all ANOVAs, fluoxetine, predation and their interaction term were used as fixed factors, and the random factor tank was nested within the fluoxetine × predator interaction term (to allow the use of individual in each replicate tank). In case of significance ( $p < 0.05$ ) Tukey's post hoc tests were conducted in order to identify between which treatments the effects were. Furthermore, ANOVAs were conducted on pooled environmental data from different sampling times to explore

differences in pH, oxygen levels and conductivity between experimental treatments. All data were analysed using SPSS version 22.0.0.0 for Mac OS X.



# Results

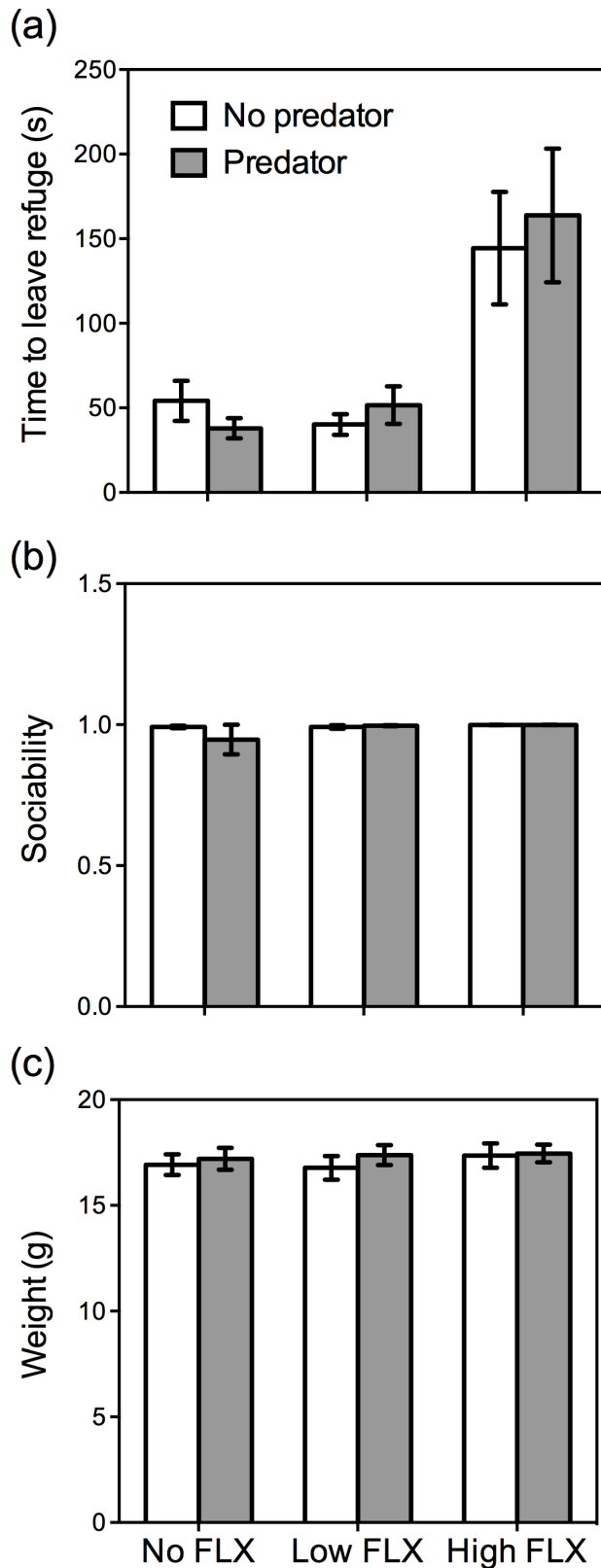
At the start of the experiment and prior to exposure, there were no significant differences between experimental treatments with regards to boldness, sociability and body weight (nested ANOVAs table 1). However, after exposure we found strong effects of fluoxetine on fish boldness (table 1 and figure 1a). Post-hoc Tukey's test revealed that fish exposed to high concentrations of fluoxetine ( $100 \mu\text{g L}^{-1}$ ), became shyer compared to unexposed individuals ( $0 \mu\text{g L}^{-1}$ ,  $p < 0.001$ ) and individuals exposed to a low concentration of fluoxetine ( $1 \mu\text{g L}^{-1}$ ,  $p < 0.001$ ). Exposure to pike had no significant effects on fish boldness (table 1 and figure 1). I found no significant effects of fluoxetine or predator exposure on fish sociability or body weight (table 1 and figure 1b-c). However, a marginally non-significant effect of predation on body weight indicated that fish exposed to a predator tended to gain more weight than fish in treatments absent of predators (table 1 and figure 1c).

Results from statistical analysis of the environmental conditions are presented in Appendix, table 1. ANOVA showed small, but highly significant variation in pH between treatments, with effect of both predator ( $p < 0.001$ ) and fluoxetine ( $p = 0.014$ ). Tukey's post hoc test revealed pH differences between  $0 \mu\text{g L}^{-1}$  and  $1 \mu\text{g L}^{-1}$  fluoxetine concentrations ( $p = 0.01$ ). Furthermore, tanks without pike had significantly higher oxygen levels than aquaria with pike ( $p = 0.002$ ). Regarding conductivity no differences were found. For more information on mean values and variation regarding environmental parameters, see Appendix table 2.

**Table 1.** Results of univariate ANOVAs presenting effects of the different treatments on fish boldness, sociability and weight. Effects of predator (with or without pike) and fluoxetine exposure (no = 0  $\mu\text{g L}^{-1}$ , low = 1  $\mu\text{g L}^{-1}$ , high = 100  $\mu\text{g L}^{-1}$ ), the interaction between those parameters and the nested effect of tank is presented. Shaded values represent post treatment data, and significant values are highlighted as bold.

Source	d.f.	m.s.	F-value	p-value
ANOVA's				
BOLDNESS				
Predator (yes/no)	1,18	0.148	0.104	0.751
	1,18	0.089	0.058	0.813
FLX (no/low/high)	2,18	0.481	0.337	0.719
	2,18	16.101	10.388	<b>&lt;0.001</b>
Predator $\times$ FLX	2,18	0.297	0.208	0.814
	2,18	0.186	0.120	0.887
Tank(Predator $\times$ FLX)	18,92	1.430	1.139	0.329
	18,92	1.555	2.769	<b>&lt;0.001</b>
SOCIABILITY				
Predator (yes/no)	1,18	20.338	0.167	0.687
	1,18	3.948	0.052	0.822
FLX (no/low/high)	2,18	16.405	0.135	0.875
	2,18	80.975	1.064	0.366
Predator $\times$ FLX	2,18	233.622	1.921	0.175
	2,18	26.632	0.350	0.709
Tank(Predator $\times$ FLX)	18,91	121.532	0.927	0.549
	18,92	76.101	0.939	0.535
WEIGHT				
Predator (yes/no)	1,19	0.004	1.661	0.213
	1,19	0.013	3.612	0.073
FLX (no/low/high)	2,19	0.002	0.833	0.450
	2,19	0.006	1.688	0.211
Predator $\times$ FLX	2,19	2.072*E-5	0.008	0.992
	2,19	0.002	0.559	0.581
Tank(Predator $\times$ FLX)	18,92	0.002	0.151	1.000
	18,92	0.003	0.176	1.000





**Figure 1.** The mean responses of crucian carps exposed to six different treatments: with or without predator and with different fluoxetine water concentrations (no FLX = 0  $\mu\text{g L}^{-1}$ , low FLX = 1  $\mu\text{g L}^{-1}$ , high FLX = 100  $\mu\text{g L}^{-1}$ ). Behaviours measured are a) boldness and b) sociability (proportion of time spent in shoaling zone); morphological response measured is c) weight. Error bars 1 SE.



# Discussion

I found that exposure to fluoxetine has the potential to alter fish behaviour. At the start of the experiment and prior to exposure, there were no differences in any of the traits measured between experimental treatments. However, after exposure, crucian carps exposed to the highest concentration of fluoxetine at  $100 \mu\text{g L}^{-1}$  significantly reduced their boldness, as evidenced by increased time to emerge from a refuge (table 1 and figure 1). My first major result was thus in line with my initial hypothesis; exposure to commonly prescribed antidepressant drugs that often enter waterways, here fluoxetine, has the potential to alter a key behavioural trait, known to influence fitness and survival (Hulthén et al. 2014; Brown et al. 2005). This result is also in line with previous studies, which have shown behavioural alterations in fish, exposed to SSRIs like fluoxetine in the range of  $0.54 \mu\text{g L}^{-1}$  –  $300 \mu\text{g L}^{-1}$  (e.g. Dzieweczynski & Hebert 2012; Winder et al. 2012).

Although fluoxetine exposure altered boldness, I could not detect any effects on sociability, highlighting that fluoxetine not necessarily targets all traits in the behavioural repertoire of crucian carps. Furthermore and contradictory to my initial prediction, I found no interactive effects of multiple stressors (i.e. simultaneous exposure to fluoxetine and a predator), nor any effects of predator exposure on carp boldness or sociability. Numerous previous studies both in laboratory and field have shown increased boldness as a result of predator exposure (e.g. Hulthén et al. 2014; Brown et al. 2005). However, these studies have been on fish in the wild constantly exposed to predators and on fish exposed for several months in the laboratory. Hence, one might argue that although behaviours often respond relatively rapidly to current conditions, the exposure time in the present experiment was too short to induce strong behavioural alterations in response to being exposed to increased risk. Similarly, the lack of response in the physiological trait body weight to the two stressors both separately and combined, might be explained by the relatively short exposure time. The nearly significant difference between carps held with or without pike indicates that there are potential effects on weight, like previous studies have reported (e.g. Hulthén et al. 2014). Therefore, for future studies I suggest longer exposure times in order to dismiss or confirm any potential effects on the examined traits of predator and fluoxetine exposure.

Why only some, but not all traits are affected by fluoxetine needs careful thought, as it can be of vital importance for the choice of traits studied and the conclusions drawn in future studies. In the case of sociability it might be so that it is a too strongly expressed anti-predatory behaviour, due to the ecology of the species. As crucian carp is very sensitive to predation and the preferred prey by pike over several other species (e.g. Brönmark et al. 1995), the urge to join a group of conspecifics for safety seems to override any potential effects of fluoxetine and predator exposure over the time period of this experiment. However, regarding boldness, when an individual crucian carp is faced with a novel environment alone, it must choose to behave more or less risky, independent of conspecifics. Since there was a significant effect on boldness from fluoxetine exposure, this can be a trait with more plasticity than sociability, which always was strongly expressed regardless of the earlier experiences of a fish. The fact that carps were tested individually in boldness trials can also have contributed to the observed differences. This reasoning however leads to further questions about the degree of sensibility of several other traits (e.g. activity and feeding), not only to the studied stressors (fluoxetine and predator exposure), but also to other in nature important stressors (e.g. lack of food, temperature fluctuations and other pollutants).

Furthermore, the effects of SSRIs are probably dependant on which species that is studied. As an example, effects on sociability have recently been shown in a fish closely related to the crucian carp, namely goldfish (*Carassius auratus*). Xie et al. (2015) found that the seven days of exposure to the SSRI sertraline decreased

sociability in this species, compared to the non-significant effect I found in this study. It is known that crucian carp is a very resilient species (Yi et al. 2007) and therefore, in order not to underestimate potential effects of pharmaceutical pollution, we also need to study more sensitive species. In order to be able to generalize on a bigger ecosystem scale from the results of laboratory studies like mine, we need to know if different naturally coexisting species respond in similar or unique ways to potential stressors. Are multiple species and competitors affected in the same direction and to the same degree, or does for example one species decrease boldness while another increase it? Are both prey and predator species affected and in that case, how? Do for example both increase their activity? First when such questions are answered, the consequences for biotic interactions at the ecosystem-level can be fully understood. To be able to predict effects on ecosystems, I therefore suggest broader studies where several species are exposed to the same experiments and treatments, so that general patterns arising from for example fluoxetine exposure can be either supported or ruled out. Furthermore, as previously proposed, a variety of traits should optimally be examined, as various traits can be of different importance to different species, depending on life-history strategies and habitat use. When designing future studies it can also be good to remember that, depending on a species ecology, different sexes and life stages can have different sensibility to stressors (e.g. Painter et al. 2009, Weinberger II & Klaper 2014) so that negative effects of pollutants are not underestimated. For example did Painter et al. (2009) show that the effects of different SSRIs in fathead minnow larvae (*Pimephales promelas*) anti-predatory behaviour depends on time of exposure, with some SSRIs disturbing behaviour to a higher degree when fish were exposed as eggs compared to as larvae. In a polluted environment, like a recipient of treated wastewater, all life stages of an organism can be expected to struggle with the same stressors and thus all should be studied if consequences of chronic exposures are to be addressed.

The ecological importance of my results are discussable since the, so far to my knowledge, highest detected concentration of fluoxetine in nature is  $0.54 \mu\text{g L}^{-1}$  (Mennigen et al. 2011). Not even the summed concentration of several SSRIs has yet been reported to be higher than  $3.2 \mu\text{g L}^{-1}$  (Mennigen et al. 2011) and thus one could argue that the highest exposure causing behavioural alterations in this experiment is not likely to happen in nature. However, numerous other studies have shown effects of environmental concentrations of SSRIs on important traits in several species (e.g. Dziewczynski & Hebert 2012; Painter et al. 2009), and negative impacts on biota cannot be excluded. In my study a very robust species was exposed and yet effects were evident after relatively short exposure times, compared to the more chronic exposure expected in nature. There are also indications of synergistic effects of several SSRIs combined (Cheer & Goa 2001), but still huge knowledge gaps regarding such effects in nature. It is also worth to note that not all of the supplied fluoxetine is bioavailable and taken up by organisms, and several factors can have had impact on actual exposure levels in my experiment. Analysis of water and fish tissue samples would shed further light on actual exposure concentrations, both in laboratory experiments and in field studies. For example did Gaworecki and Klaine (2008) only find 67% of their supplied fluoxetine in the water, speculating about absorption to organic matter, plastic details and the aquaria glass walls themselves, photolysis, hydrolysis and oxidation. Uptake and toxicity of fluoxetine is also known to depend on pH, since ionizing of the substance in environments with pH differing from its  $pK_a$ -value makes it less lipophilic and less bioavailable (Nakamura et al. 2008; Boström & Berglund 2015).

With this in consideration, it is also interesting to note that there seems to be effects of pike presence on pH and oxygen levels in the aquaria (Appendix, table 1), probably simply due to pike excreting faeces and consuming oxygen. This is something that can influence the results of laboratory projects such as mine, since these environmental parameters can directly influence test subjects health. The difference in oxygen levels was small and the aquaria were more or less oxygenated to maximum dissolution why this difference between treatments should not severely impact crucian carps, known as a species with high tolerance towards abiotic disturbances like hypoxia (Holopainen et al. 1997b). However, the lower pH in aquaria with pike needs more careful thought, since pH influences the proportion of fluoxetine that is ionized. The differences in pH between aquaria with and without pike was small, but still statistically significant (Appendix, table 1), why presence of pike indirectly can have decreased the amount of bioavailable fluoxetine. In addition, pike can also be expected to take up part of the bioavailable fluoxetine and thereby

directly reduce the amount available for carp uptake. To minimize the impact of predators sharing the aquaria of some carps but not others, only predator cues could have been added instead of live predators. However, since my results showed no significant effects of pike on any of the measured parameters (boldness, sociability and body weight), and no interactions between predator and fluoxetine treatments were found, this scenario is unlikely. The nearly significant effect of predator on body weight is probably part of the regular well-known inducible morphological defence that crucian carp possess (e.g. Brönmark & Pettersson 1994; Hulthén et al. 2014), and not due to indirect effects of pike presence on pH and oxygen levels in aquaria. Crucian carp exposed to predators decrease activity levels and hence, have more energy to allocate to growth (Johansson & Andersson 2009).

One should also remember that the pathways in which SSRIs act still are not completely understood. For example, according to previous work, there appears to be “feed back mechanisms” counteracting the increased levels of serotonin in synaptic clefts resulting from SSRIs. Gaworecki and Klaine (2008) reported a decrease in serotonin release from the pre-synaptic cells in hybrid striped bass (*Morone saxatilis* × *M. chrysops*) after only six days of fluoxetine exposure. They hypothesize that this is due to activation of certain receptors counteracting the reuptake inhibitive role of SSRIs. Daily samples of fluoxetine and serotonin levels in fish would further increase the understanding of the drug response and time-windows when effects are most pronounced, but this was neither practically doable nor the aim of my project. However, more knowledge on this subject is required in order for us to evaluate the importance of feedback mechanisms.



# Conclusions

My findings show that high concentrations of the anti-depressant fluoxetine have impact on crucian carp boldness, but contradictory to my predictions I found no effects on the observed traits (boldness, sociability, body weight) of predator exposure, nor any interaction between the two stressors fluoxetine and predator exposure. However, as the problem of pollutants in aquatic environments is complex, I stress the need for further investigations of effects on more key traits in several species of different trophic levels, in order to identify potential risks of pollution of fresh water ecosystems, so that society get the chance to foresee and prevent negative consequences. Studies of effects on biota of all pollutants singularly, and perhaps most importantly, combined with each other and other stressors are vital when it comes to understanding and preventing negative environmental impact of WWTPs.

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# Appendix

**Table 1.** Results of univariate ANOVAs comparing different treatments regarding environmental parameters a) pH, b) oxygen levels and c) conductivity. Significant values are highlighted as bold.

Source	d.f.	m.s.	<i>F</i> -value	<i>p</i> -value
ANOVA's				
pH				
Predator (yes/no)	1,18	0.769	72.560	<b>&lt;0.001</b>
FLX (no/low/high)	2,18	0.057	5.409	<b>0.014</b>
Predator × FLX	2,18	0.019	1.805	0.193
Tank(Predator × FLX)	18,48	0.011	0.870	0.614
OXYGEN				
Predator (yes/no)	1,18	2.067	13.867	<b>0.002</b>
FLX (no/low/high)	2,18	0.198	1.331	0.289
Predator × FLX	2,18	0.106	0.711	0.504
Tank(Predator × FLX)	18,48	0.149	14.907	<b>&lt;0.001</b>
CONDUCTIVITY				
Predator (yes/no)	1,18	329.389	1.721	0.206
FLX (no/low/high)	2,18	395.504	2.067	0.156
Predator × FLX	2,18	137.371	0.718	0.501
Tank(Predator × FLX)	18,48	191.384	1.318	0.219

**Table 2.** Mean values of pH, oxygen and conductivity data with one standard error for the different treatments.

TREATMENT	pH	Oxygen (mg L <sup>-1</sup> )	Conductivity (μS cm <sup>-1</sup> )
No predator, no FLX	7.63 ± 0.033	8.66 ± 0.036	207.03 ± 5.788
No predator, low FLX	7.74 ± 0.025	8.71 ± 0.023	196.94 ± 2.445
No predator, high FLX	7.74 ± 0.038	8.65 ± 0.049	195.03 ± 2.654
Predator, no FLX	7.47 ± 0.032	8.38 ± 0.084	205.83 ± 3.273
Predator, low FLX	7.55 ± 0.029	8.46 ± 0.056	203.33 ± 3.322
Predator, high FLX	7.47 ± 0.030	8.16 ± 0.098	202.67 ± 3.258



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