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The impact of the antidepressant fluoxetine on personality traits in the isopod *Asellus aquaticus*

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Titel
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Impact of the antidepressant fluoxetine on behaviour on the isopod *Asellus aquaticus*

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Sammanfattning
Abstract

Pharmaceuticals that end up in our aquatic environment continue to increase. In recent years, serotonin re-uptake inhibitors (SSRI) have increased in usage as it is considered safer than other substances to treat depression. Fluoxetine (Prozac) is a widely used anti-depressant that commonly leak out after human use to aquatic environments. Although widely spread, the impact of fluoxetine on aquatic animals in is poorly investigated. The objective of this study was to see if fluoxetine impacts the behaviour of freshwater isopod *Asellus aquaticus. Asellus aquaticus* were exposed to an ecologically relevant concentration of fluoxetine for 28 days. Through a series of behavioural assays designed to measure the personality traits boldness, activity, exploration and escape behaviour, *Asellus aquaticus* responses were investigated. *A. aquaticus* can differ greatly in phenotype, from non-pigmentation to dark pigmentation. Further objective was therefore to investigate if pigmentation correlated with any of the measured behavioural responses, due to potential across-reaction between serotonergic and melatoneric systems. I found that fluoxetine reduced boldness, but had no effect on activity, exploration or escape behaviour. Furthermore, I observed no correlation between pigmentation and behaviour measured in fluoxetine exposed, or control animals. These results indicate that fluoxetine at low levels affect boldness of wild *A. aquaticus* but no other personality traits explored. However, other research contradicts these results and show that fluoxetine can affect a range of behaviours. Taken together fluoxetine can have ecological impact on aquatic environments. Hence, our residual pharmaceuticals can have ranging effects.

Nyckelord
Keyword
Asellus aquaticus, Behaviour, Fluoxetine, Serotonin

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1. Abstract

Pharmaceuticals that end up in our aquatic environment continue to increase. In recent years, serotonin re-uptake inhibitors (SSRI) have increased in usage due to its use to treat depression. Fluoxetine (Prozac) is a widely used human anti-depressant that commonly leak out to aquatic environments. Although widely spread, the impact of fluoxetine on aquatic animals is poorly investigated. The objective of this study was to investigate if exposure to fluoxetine impacts the behaviour of freshwater isopod *Asellus aquaticus.* I did this by exposing *A. aquaticus* to an ecologically relevant concentration of the fluoxetine for 28 days. Through a series of behavioural assays, the personality traits boldness, activity, exploration and fear were measured. *A. aquaticus* can differ greatly in phenotype, from non-pigmentation to dark pigmentation. I therefore also aimed to investigate if pigmentation interacted with any of the measured behaviour, due to potential cross-reaction between serotonergic and melatonergic systems. I found that fluoxetine reduced boldness, but had no effect on activity, exploration or fear responses. Furthermore, I observed no correlation between pigmentation and behaviour measured in fluoxetine exposed, or control animals. These results indicate that fluoxetine at low levels affect boldness of wild *A. aquaticus*. Fluoxetine could however not be seen to affect the other three personality traits tested, in this case exploration, activity and fear. However, other studies contradicts these results and show that fluoxetine can affect a range of behaviours. Taken together, fluoxetine can have ecological impact on *A. aquaticus* and hence, our residual pharmaceuticals can have ecologically important effects.

2. Introduction

Freshwater environments receive a range of contaminants through human activity. One group of contaminants that has increased in the environment is pharmaceuticals. Wastewater treatment plants can only remove pharmaceuticals to a certain extent. As a result, pharmaceuticals reach water bodies through their emission points (Swedish Environmental Protection Agency 2017).

One group of pharmaceuticals that in recent years has attracted increased interest is Pharmaceutically Active Compounds (PhACs) (De Castro-Català et al 2017). Antidepressants are PhACs that have increased in both usage and in the environment the past decade. Moreover, PhACs ecotoxicological effects are not fully understood (Bossus et al 2014). Presence of antidepressants in the environment is a potential problem, the reason being that its active substances are made to alter hormone levels and signal substances in humans and could have effects on aquatic and marine organisms too. Adding to the concerns, antidepressants have the ability to bioaccumulate and persist for a long time in aquatic environments (reviewed by Pereira et al 2015). This is confirmed by the fact that PhACs have been found in many different taxa (Bean et al 2014; Gonzalez-Rey & Bebianno 2013; Richmond et al 2016). This study will focus on a specific group of PhAC called Selective Serotonin Re-uptake Inhibitors (SSRIs). SSRIs treats depression disorders and is one of the most prescribed groups of anti-depressants. SSRIs block nerve cells´ ability to absorb serotonin, resulting in an increased stimulation at the post synaptic nerve (Stahl 1998).

Active forms of antidepressants, especially serotonin re-uptake inhibitors (SSRIs), are of concern when they leak out in nature as they potentially have disrupting effects on non-target species (Gonzalez-Rey & Bebianno 2013). Serotonin controls functions within both invertebrates and vertebrates, such as behaviour, metabolism and reproduction, which suggests that SSRIs can affect non-target organisms (Bossus et al 2014). Given the importance of serotonin in many organisms, common SSRIs in our environment should be investigated and their ecological impact clarified.

Fluoxetine, commonly known as Prozac, is an antidepressant and SSRI that is widely used and can be found in effluent from wastewater treatment plants. Fluoxetine has the potential to alter ecosystem processes such as metabolism, biofilm and biomass (Richmond et al 2016). Fluoxetine's non-target effects can be found in a range of organisms e.g. gill damage in bivalvies (Gonzalez-Rey & Bebianno, 2013), disturbance of the endocrine system in Zebrafish (*Danio rerio,* Meshalkina et al 2018) and behaviour responses in crustaceans (Tierney et al 2004; Bossus et al 2014; De Castro-Català et al 2017). Yet, what impact ecologically relevant concentrations of fluoxetine have on ecosystems is poorly investigated. In Sweden, higher concentrations than 50 ng/L fluoxetine have not been registered in outgoing water from wastewater treatment plants (Woldegiorgis et al 2007). Investigating an ecologically relevant

concentration of fluoxetine on an organisms important to its ecosystem, could give an insight in what environmental impact can fluoxetine have.

Asellus aquaticus is an invertebrate that is important to the freshwater environments, as it degrades organic material and is a food source for larger predators (Eroukhmanoff et al 2009; Rask & Hiisivuori 1985). *A. aquaticus* is a common benthic organism in temperate climates in the northern hemisphere. It has also been established that *A. aquaticus* frequency in the phenotype pigmentation can differ greatly, depending on habitat (Hargeby et al 2004).

An organism's behaviour in ecological and evolutionary contexts can influence an individual's fitness (Saaristo et al 2017). Of increased interest in animal behaviour research lately, is behaviour that describes variation in animal personality (i.e. among-individual consistency in behaviour, Dall et al 2004, Réale et al 2007). Animal personality is described in a broad range of species (Gosling 2001), and shown to have ecological and evolutionary consequences for the individual. I therefore focused on behaviour used to describe variation in the personality traits boldness, exploration, activity and fear responses (Réale et al 2007).

Studying fluoxetine's effect on *A. aquaticus* behaviours can provide information and basis for future risk-assessments and give an ecological perspective on SSRIs effect on organisms. Furthermore, an organism's behavioural traits, can combined give information on how well *A. aquaticus* interacts with a new environment (Bean et al 2014). Studying pharmaceutical's effect on ecosystems is crucial to be able to protect vulnerable environments and, if significant, work to regulate the contamination. Without knowledge about anthropogenic substances hazardness, future environmental effects will not be prevented. Therefore, the aim of this study is to investigate the effect of anti-depressants on an aquatic organism to detect possible negative effects.

2. Materials and methods

2.1. Study species

Wild caught *Asellus aquaticus* were used in this research project. *A. aquaticus* is a crustacean belonging to the order isopoda that lives in freshwater environments. The species is distributed throughout temperate climates, Europe (Including all of Russia) and North America (Verovnik et al 2005). Individuals in this study were caught in lake Tåkern (58°21 N, 14°50 E), Östergötaland county, Sweden. This was done by harvesting *Chara tomentosa* from shallow water 1-2 meters depth, on the date 2019-03-21. *Chara tomentosa* is a submerged stonewort, and a common habitat for *A .aquaticus* in Lake Tåkern (Eroukhmanoff et al 2011).

A. aquaticus inhabiting lake Tåkern differs in pigmentation. Individuals that inhabit the green algae *Chara spp.* have a lighter phenotype than those living on reeds (*Phragmites australis)* (Hargeby et al 2004). This is likely a response to the change in the local environment that lake Tåkern has seen the past years (Blindow 2011). Due to a dramatic shift from a phytoplankton dominant state to a macrophyte dominant state in Lake Tåkern, *Chara spp*. started to colonise the sediment areas in the limnetic zone (Hargeby et al 2004). *A. aquaticus* in the new habitat (*Chara spp.)* became lighter in their appearance. *A. aquaticus* has a ommochrome-based pigmentation, where the amino acid tryptophan is the precursor (Lürig et al 2019). Serotonin is known for its complex effects on organisms and can influence a range of melatonergic processes. There is considerable proof that serotonin can influence the melatoneric system (Duck et al 2012; Liao et al 2012). This could mean that fluoxetine which increases serotonin levels, also could interfere with *A. aquaticus* pigmentation.

Isopods were held in a plastic tank (40cm x 13cm x 17cm) and kept in 5 °C for 5 days. They were later sorted into smaller tanks (16cm x 18.5cm x 14cm), which were filled with two litres of water from lake Tåkern. Sorting was done by pigmentation. Dark, intermediate and light individuals were separated arbitrarily, this was done to ensure that the caught *A. aquaticus* had a variate pigmentation. The sex of *A. aquaticus* was distinguished either by the pre-copulatory stage, where the male locks on to a female, thus the individual that is positioned underneath is the female, or by the presence of an oviduct pouch (Hargeby et al 2004). Not more than 16 individuals were sorted into each plastic bin mainly to minimize potential stress in the isopods. Isopods were fed leaves (*Alnus glutinosa* and *Ulmus glabra*) that were colonized (growth of bacteria on organic compound) for three weeks. Leaves also work as a refuge for *A. aquaticus*, since isopods usually want something to hold on to. To keep the water oxygenated, a construction of air pipes was put into the water and kept on during the hole exposure period.

2.2.Exposure to fluoxetine

Isopods where sorted into ; exposed males, exposed females, male control and female control. There were two treatment groups; Control (water from lake Tåkern), and exposure to fluoxetine (20 ng/L). The fluoxetine concentration used in this study refers to the concentration found in the surface water of lake Roxen, Linköping, where 20 ng/L was discovered in surface water in 2008 (Helmfrid & Eriksson 2010). No data on pharmaceuticals in lake Tåkern could be found.

Fluoxetine was added from a stock solution to half of the plastic tanks. The fluoxetine solution (1 mg/L) was prepared with deionised water and fluoxetine hydrochloride (Crystal form, Sigma-Aldrich). Water in the plastic tanks was changed once a week during the whole exposure period which was 28 days.

2.3. Behavioural assays

Isopods that took part in the behavioural assays (see below) were chosen at random. An individual that was chosen did all behavioural assays in certain order: Boldness, exploration, activity and escape behaviour. All the behavioural assays were set to a time limit of 5 minutes, if the individual exceeded this time it got the maximum score (Harris et al 2011; Neill et al 2018). The individual was allowed to rest for 5 minutes between the behavioural assays to minimize stress (Neill et al 2018). Pigmentation of individual isopods was scored between 1-20: 1 was 100% white and every step decreased whiteness by 5%. The grey-scale was made in Adobe-photoshop Cs5

2.3.1. Boldness

Boldness in organisms can be studied by investigating an individual's risk taking behaviour (reviewed by Réale et al 2007). This is often done by providing the study organism with a some sort of refuge in a constructed arena, and measuring the time it takes for the organism to leave the refuge (Bevan et al 2018; Harris et al 2011; Réale et al 2007; Tremmel & Müller 2012). The arena consisted of a plastic petri dish (ø 13.6 cm), placed in the middle was a refuge consisting of a small folded wooden stick (*Ulmus galabra*). Exposed *A. aquaticus* did their trials in water from lake Tåkern with fluoxetine (20 ng/L), while control groups had uncontaminated water from lake Tåkern. The petri dish was filled with 15 mm of water. Before the trial began, an opaque tube was placed over the refuge, this served as a acclimation unit and to make the refuge more easily found by the isopod (Harris et al 2011). The isopod was then moved from the plastic tank into the tube covering the refuge where the isopod could acclimatise and seek refuge for 1 minute. The opaque tube was then lifted, and the time it took for the isopod to leave the refuge was measured to investigate *A. aquaticus* boldness. The isopod had to let go with all its limb to be recognized as a fulfilled departure from the refuge. A limit of 5 minutes was set and isopods that would not leave the refuge was given the maximum score of 300 seconds.

2.3.2. Exploration

Exploration test measure the time it takes for an individual to visit all zones in a novel arena, commonly known as an open field test. This is a recognized way of measuring exploratory behaviour (Perals et al 2017; Tremmel & Müller 2012). In this case the same petri dish was used as in the boldness test, but this time divided into 8 equally sized zones. *A. aquaticus* was allowed to acclimatise for 1-minute. The whole body of the isopod had to cross the marked zone to be counted as a new visit. The test had a limit of 5 minutes, and if the isopod had not visited all the zones during this time it was given the maximum score of 300 seconds.

2.3.3. Activity

To measure the general activity the main goal was to record the isopods total time moving. This is commonly investigated by recording the total area covered and the study organisms overall movement (Réale et al 2007; Sinyakova et al 2018). In this case, the same petri dish as in the exploration test was used (see above). The number of times the isopod changed zones was recorded during a 5-minute period. As in the exploration test, the whole-body hade to cross the line to be counted as a new visit. *A. aquaticus* was allowed to acclimatise for 1 minute.

2.3.4. Escape behaviour

To investigate fear in the isopods a simulated predator attack is recognized as a tool of measurement (Eroukhmanoff & Svensson 2009; Harris et al 2011). The isopod was moved into the petri dish where it acclimatized for 1 minute. A plastic rod was then used to poke the isopod gently on the back for 10 seconds, thus simulating a predator attack. After the simulation ceased, two measurements were investigated: (i) time spent moving (ii) how long it stayed in a 'freezing' state after the initial movement. 'Freezing', or sudden immobilization, is an anti-predator behaviour which can be seen in a range of organisms (Saaristo et al 2017). The test had a maximum time limit of 5 minutes.

3. Statistical analysis

The results from the behavioural response tests were not normally distributed, so non-parametric statistical tests were used.

To investigate if there was a correlation between *A. aquaticus* pigmentation and the response variables measured (latency to leave refuge, exploration, activity and escape behaviour), Spearman's rank correlation tests were used.

To examine if fluoxetine had an effect on *A. aquaticus* behaviour, Kruskal-Wallis one-way analysis was used. The test compered the 4 different groups (male fluoxetine, male control, female fluoxetine and female control). If the test results showed a difference, a pairwise analysis was made to see which groups that differed ($P < 0.05$). All analyses were done in the statistical analyse programme IBM SPSS (version 25).

4. Results

4.1. Correlation between pigmentation and behaviour

There was no correlation between *A. aquaticus* pigmentation and any of the behavioural responses, latency to leave refuge, exploration, activity and escape behaviour (moving/freezing) (table 1).

Table 1. The relationship between pigmentation and behaviour in *Asellus Aquaticus*. *Correlation between the responses. Boldness (latency to leave refuge, in seconds), exploration (visiting all different zones, in seconds), activity (changing of zones), moving (seconds) and freezing (seconds). N = 20. (M/F = Male Fluoxetine, M/C = Male Control, F/F = Female Fluoxetine, F/C = Female Control). r_s = correlation coefficient from Spearman rank correlation test.*

	Boldness	**Exploration**	**Activity**	**Moving**	**Freezing**
M/F	r_s= 0.27	r_2 = 0.16	r_s= - 0.22	r_s = 0.00	r_s = -0.34
	P = 0.26	P = 0.51	P = 0.35	P = 0.99	P = 0.15
M/C	r_s = - 0.12	r_2 = -0.02	r_s = 0.05	r_s = 0.20	r_s = 0.24
	P = 0.62	P = 0.92	P = 0.83	P = 0.41	P = 0.30
F/F	r_s = -0.02	r_s = -0.05	r_s = 0.13	r_s = -0.29	r_s = -0.34
	P = 0.92	P = 0.85	P = 0.60	P = 0.23	P = 0.15
F/C	r_s = -0.31	r_s = -0.33	r_s = 0.32	r_s = -0.18	r_s = 0.04
	P = 0.18	P = 0.15	P = 0.16	P = 0.46	P = 0.88

4.2. Boldness

A difference could be seen between the two male treatment groups in boldness (H_3= 43.9, P = 0.000). Exposed males often stayed at the refuge compared to the control group. (Male fluoxetine: mean ± SE: 133.16 ± 31.40: Male control: mean ± SE: 23.27 ± 14.99). There was a difference between male and female groups (see table 2)

Table 2. Boldness comparison between fluoxetine exposed individuals and control. *Pairwise Kruskal-Wallis results compering 2 treatment groups. Exposed males/females and their control groups, N = 80. Where the time it took an individual to leave the constructed refuge was measured*.

	p
Male fluoxetine vs. Male control	**0.03**
Female fluoxetine vs. Female control	1.00
Male fluoxetine vs. Female fluoxetine	0.42
Male control vs. Female control	**0.00**
Male fluoxetine vs. Female control	0.05
Male control vs. Female fluoxetine	**0.00**

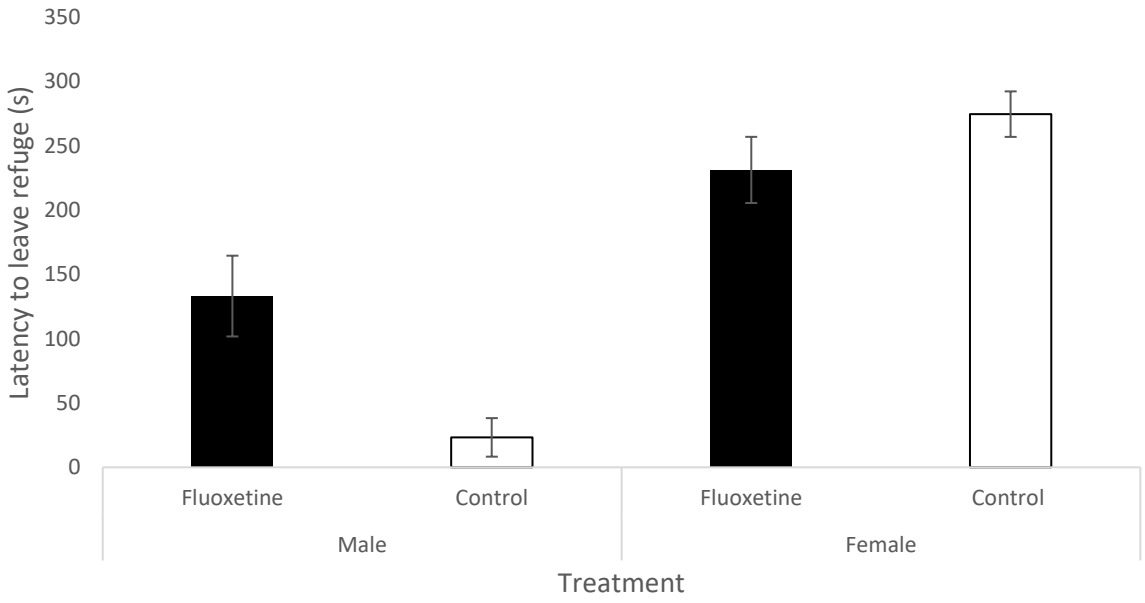

Figure 1. Boldness in *Asellus aquaticus* exposed (black) and not (white) to fluoxetine. *The amount of time (seconds) it took to leave a constructed refuge in an experimental arena. Columns show mean ± SE. Each group N = 20 (Total N = 80*).

4.2. Exploration

Fluoxetine did not have an effect on *A. aquaticus'* exploratory behaviour. Moreover, sex did only show a difference between exposed males and the female control group (Male fluoxetine: mean ± SE: 63.2 ± 9.1: Female control: mean ± SE: 180.86 ± 25.86, table 3). Exposed males and females tended to differ (, Male fluoxetine: mean ± SE: Female fluoxetine: mean ± SE: ±, table 3).

Table 3. Exploration comparison between fluoxetine exposed individuals and control. *Pairwise Kruskal-Wallis results compering 4 treatment groups (Exposed males/females and their control groups), N = 80. Where the amount of time it took an individual to visit all zones was observed.*

	p
Male fluoxetine vs. Male control	1.00
Female fluoxetine vs. Female control	1.00
Male fluoxetine vs. Female fluoxetine	0.08
Male control vs. Female control	0.34
Male fluoxetine vs. Female control	**0.01**
Male control vs. Female fluoxetine	1.00

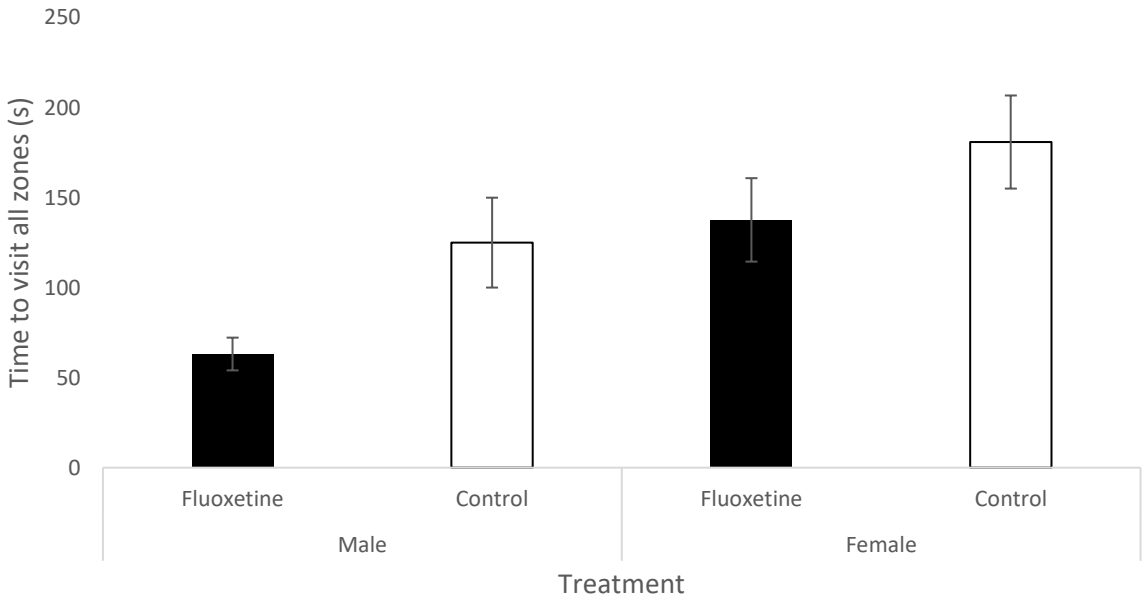

Figure 2. Exploration in *Asellus aqaticus* exposed (black) and not (white) to fluoxetine. *Time it took (seconds) A. aquaticus to visit all the zones in an experimental arena. Columns show mean ± SE. Each group n = 20 (total n = 80). Black chart bars show individuals exposed to fluoxetine. White chart stacks show control groups not exposed to fluoxetine.*

4.3. Activity

Kruskal-Wallis test showed that there was a difference between the groups (H_3=15.03, P = 0.02). However, this was between exposed males and control females (Male fluoxetine: mean ± SE: 42.85 ± 3.32: Female control: mean ± SE: 20.20 ± 4.68). There was no difference in

general activity between exposed males and its control group or the exposed females and their control group.

Table 4. Activity comparison between fluoxetine exposed individuals and control *Pairwise Kruskal-Wallis results compering 2 treatment groups. Exposed males/females and their control groups, N = 80, P < 0.05. The test observed how many times A. aquaticus changed zones for 5 minutes*.

	p
Male fluoxetine vs. Male control	0.65
Female fluoxetine vs. Female control	1.00
Male fluoxetine vs. Female fluoxetine	0.06
Male control vs. Female control	0.19
Male fluoxetine vs. Female control	**0.00**
Male control vs. Female fluoxetine	1.00

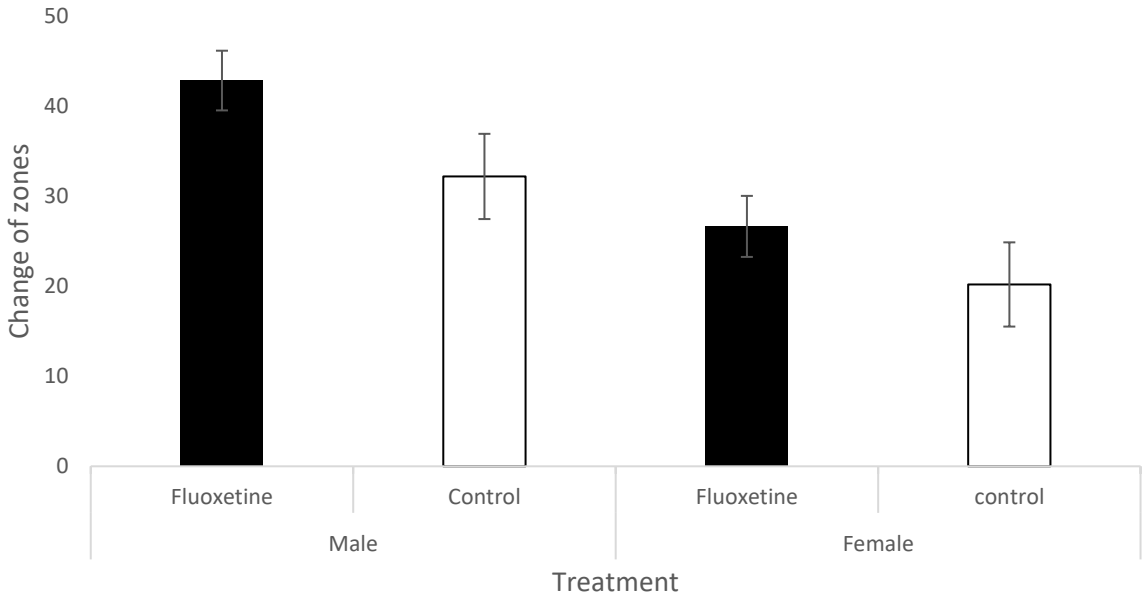

Figure 3. Activity in *Asellus aqaticus* exposed (black) and not (white) to fluoxetine. *The amount of times A. aquaticus changed zones during a 5-minute period. Columns show mean ±SE. Each group n = 20 (total n = 80). Black chart bars show individuals exposed to fluoxetine. White chart stacks show control groups not exposed to fluoxetine.*

4.3.1. Freezing

Kruskal-Wallis test showed no difference between exposed and unexposed *A. aquaticus.* The results also showed that the exposed females kept a freezing position longest. There is a

difference between exposed females and both male groups. Overall the females kept a freezing position longer then the males.

Table 5. Freezing; Comparison between fluoxetine exposed individuals and control. *Pairwise Kruskal-Wallis results compering 2 treatment groups. Exposed males/females and their control groups, N = 80, P < 0.05. Where the A. aquaticus time spent in a freezing position was measured*.

	p
Male fluoxetine vs. Male control	1.00
Female fluoxetine vs. Female control	0.23
Male fluoxetine vs. Female fluoxetine	**0.03**
Male control vs. Female control	1.00
Male fluoxetine vs. Female control	1.00
Male control vs. Female fluoxetine	**0.03**

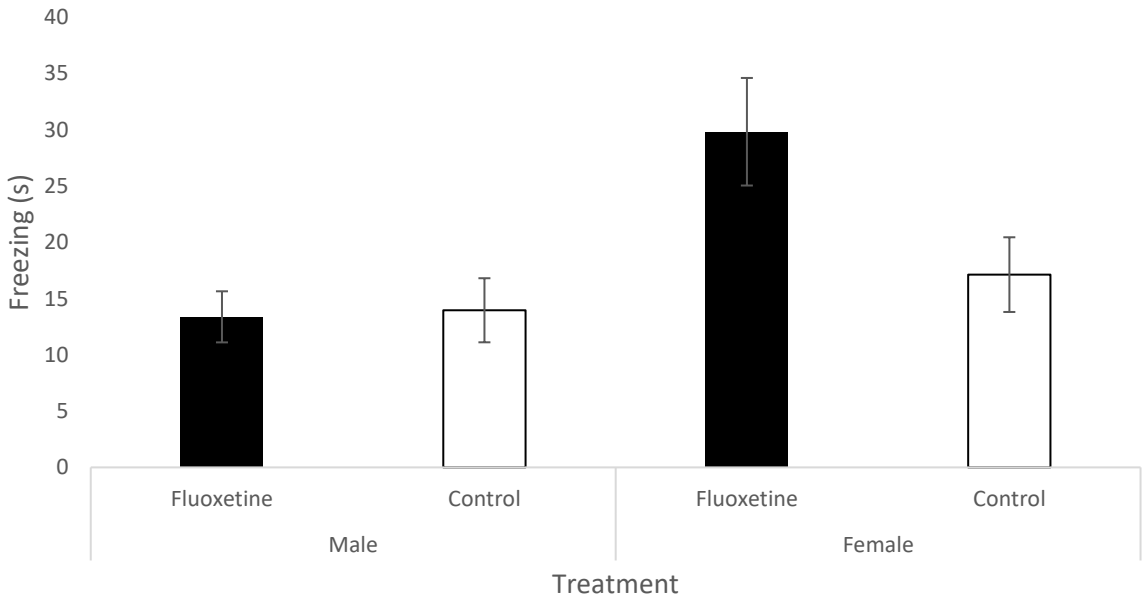

Figure 4. Freezing in *Asellus aqaticus* exposed (black) and not (white) to fluoxetine. *Shows the amount of time (seconds) the A. aquaticus kept a freezing position after predator attack. Columns show mean +-SE. Each group had n = 20 (total of n = 80). Black chart stacks show individuals exposed to fluoxetine. White chart bars show control groups not exposed to fluoxetine.*

4.3.2. Moving

Kruskal-Wallis test showed no difference between any of the 4 groups ($H_3 = 4.161$, $P = 0.245$).

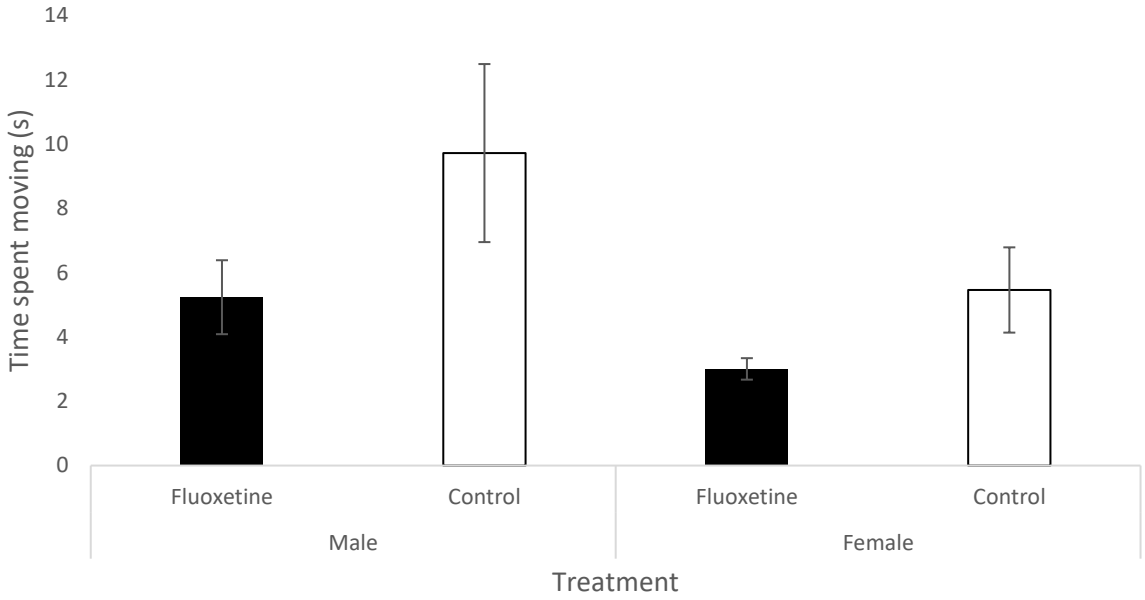

Figure 5: Moving in *Asellus aqaticus* exposed (black) and not (white) to fluoxetine. *The total time (seconds) A. aquaticus spent moving after a simulated predator attack. Columns shows the mean +- SE. Each group n = 20 (total n = 80). Black chart stacks show individuals exposed to fluoxetine. White chart stacks show control groups not exposed to fluoxetine.*

5. Discussion

The aim of this study was to test an ecologically relevant concentration of an anti-depressant substance, in this case 20ng/L of the serotonin re-uptake inhibitor fluoxetine , to see if this had any effect on personality in a common aquatic organism. In this case *A. aquaticus*, a decomposer and important prey in aquatic environments (Hargeby et al 2005; Rask & Hiisivuori 1985). I did this by exposing *Asellus aquaticus* to fluoxetine for 28 days and compered them with animals not exposed, so-called controls in behavioural assays measuring boldness, exploration, activity and fear. At the same time, *A. aquaticus* pigmentation, and how this effected *A. aquaticus* behaviour was investigated. This was done by determining their pigmentation using a grey-scale and then investigate if pigmentation interacted with any of the measured behaviours. The aim for this part of the work was to see if there was any across-reaction between serotonergic and melatonergic systems. In this study, one behavioural assay showed that there was a difference between exposed and unexposed individuals. This was the boldness test, where fluoxetine exposed males stayed longer at the refuge. These results indicate that fluoxetine at the concentration of 20 ng/L has a disrupting effect on *A. aquaticus* when it comes to boldness.

In recent years, studies on smaller crustaceans suggest that fluoxetine could influence behavioural responses and ultimately affect fitness (Bossus et al 2014; De Castro-Català et al 2017; Sinyakova et al 2018). There have also been noticeable changes in behaviour at low concentrations, in this case low concentrations is referred to a span between 0 – 100 ng/L (De Castro-Català et al 2017). Even though *A. aquaticus* is a well-studied organism in ecological research (Eroukhmanoff et al 2011; Harris et al 2011; Johansson 2005; Mccallum 2019), no studies could be found that investigates SSRIs impact on *A. aquaticus*, which in turn makes it more difficult to find information about *A. aquaticus'* sensitivity to fluoxetine or SSRIs. However, studies on the amphipod *Gammarus pulex*, where the organisms were exposed to 100ng/L fluoxetine altered *G. pulex'* swimming velocity, and lowered their feeding rate (De Castro-Català et al 2017). Moreover, *Echinogammarus marinus*, another amphipod, showed an increased velocity in swimming during acute exposure to fluoxetine at 100 ng/L and 10ng/L, respectively (Bossus et al 2014). As *A. aquaticus* is a close relative to these small crustaceans, one can suspect that *A. aquaticus* shows similar behavioural changes and syndromes. In Sweden, concentrations as high as 100ng/L fluoxetine have not been found in either wastewater treatment plants or lake surface waters (Woldegiorgis et al 2007), but it is the reality in other parts of Europe, where effluents from wastewater treatment plants have been measured to have fluoxetine concentrations close to 1.4 µg/L fluoxetine (Munch-Christensen 2009). However, mentioned studies give proof that organisms get affected and open discussions on how sensitive common freshwater and marine invertebrates are. When reviewing studies on various organisms like fish, molluscs and crustaceans, it was found that fluoxetine usually increases activity, makes organisms more fearless to predator threats, alters exploratory behaviours and in some cases causes gill damage (Gonzalez-Rey & Bebianno, 2013; Saaristo et al 2017). Accordingly, my prediction was that *A. aquaticus* would show

somewhat similar tendencies, meaning higher tendency to leave refuge, higher activity and decreased time moving after a simulated predator attack.

There was no correlation between pigmentation and measured behaviour. The focus of this test was to investigate if lighter or darker individuals were influenced differently by fluoxetine, as their pigmentation pathway has a similar pathway as that of serotonin (Liao et al 2012), which fluoxetine affects. Fluoxetine could potentially alter both activity and levels of different enzymes, which in turn could cause a shift in biochemical pathways. Fluoxetine has shown to increase both cellular tyrosinase levels and enhances tyrosinase-related proteins, which in the end leads to an increase in melanin levels (Liao et al 2012). This proves that fluoxetine has the potential to not only affect serotonin levels but also alter other physiological processes, like the melatonergic system. *A. aquaticus* pigmentation did however not correlate with any of the measured behaviours. Thus, no across-reaction between serotonergic and melatonergic system could be seen at the concentration of 20 ng/L fluoxetine. This suggests that fluoxetine or increased levels of serotonin does not affect darker or lighter individuals differently.

Although fluoxetine potentially could alter a range of biochemical pathways and affect physiological processes, it might be difficult to prove this by conducting behavioural assays, especially on wild caught individuals as other factors might influence their behaviour. As an example, *A. aquaticus* is thought to behave differently depending on what environment it inhabits. *A. aquaticus'* inhabiting reeds (*Phragmites australis*) are usually bigger and darker, and the main predator in this habitat is dragonfly larvae (*Aeshna spp*.). Meanwhile, *A. aquaticus* inhabiting stonewort (*Chara spp*.) are usually preyed upon by perch (*Perca fluviatilis*), resulting in different behavioural responses when it comes to boldness, activity, exploration and fear (Harris et al 2011). This makes it more difficult to know what a response is due to, e.g. chemical cue, assay set up or treatment.

The boldness assay showed a difference between males exposed to fluoxetine and control males. Male control emerged from refuge faster. However, Male controls seemed to be more easily startled when the opaque tube was lifted, which might have made male controls emerge from refuge faster. Fluoxetine exposed males seemed more indifferent to the opaque tube and at the same time less interested in exploring the novel environment. This brings up the question if fluoxetine did make *A. aquaticus* less bold or more indifferent to a potential threat. Moreover, females tended to stay at refuge, and usually did so during the whole 5-minute time limit. Females also put in a higher effort in hiding, which they did by crawling under the refuge where they stayed put. This result was expected, as the females in general are less exploratory (Harris et al 2011). There is also the fact that the females used in the behavioural assays carried eggs, which could result in less risk taking and more cautious attitude towards a novel environment.

The exploration test did not show a difference between exposed individuals and control groups. Interestingly, both exposed females and males visited all eight zones faster compared

to their control groups, although not significant. Fluoxetine has been proved to increase velocity and activity in crustaceans (Bossus et al 2014; De Castro-Català et al 2017), which matches these means. Accordingly, the exposed individuals, both female and males, had higher activity means. Exploration and activity assays are usually linked, commonly known as a behavioural syndrome (Schuster et al 2017). As mentioned, females are usually less exploratory than males, but size is also a factor that has not been considered in this study. Males are bigger; thus, they have longer legs and can move faster than females.

A. aquaticus escape behaviour, which was measured by time spent moving after predator attack and time spent in a freezing position after movement, did not show any difference between treatment groups. Freezing or sudden immobility is a common strategy among animals, usually to prevent detection (Saaristo et al 2017). Studies on fear in invertebrates while exposed to SSRIs could not be found, making it more difficult to hypothesise about the outcome. However, there have been more studies done on fish, where fluoxetine has proved to alter anti-predator behaviour. Wild guppies (*Poecilia reticulata)* spent longer time in a freezing position after predator attack (Saaristo et al 2017). Anti-predator behaviour is crucial for an individual's survival, making this test quite interesting in an ecological point of view. A future study could be to expose individuals over a longer time period. This would make the test even more environmentally accurate, as wild populations are constantly exposed to pharmaceuticals. A future study could also be to conduct a dose response test to see which concentrations that, in a shorter period, have an effect on *A. aquaticus*.

According to the result in this study, fluoxetine (20 ng/L) did not seem to have a major impact on *A. aquaticus* behaviour. There was however one assay that showed a difference between treatment groups, in this case boldness. It is important to remember that animal personality can be affected in different ways when exposed to substances. Meaning, even though only one response differed, this does not mean that fluoxetine is less of a concern. Personality traits in animals are not always linked and will ultimately be affected in different ways.

Behaviour in animals are complex and not easily tested. As reviewed by Gosling (2001), personality among organisms can be tested in more than one way. Thus, what tests that are best for a specific animal is not always certain. In this study, 4 well-established behavioural assays were used. The boldness test showed that male controls left refuge almost instantly after the opaque tube was lifted . As mentioned above, this boldness test might have caused unexposed individuals to be more startled, opening a discussion if this test actually tested fear and not an individual´s boldness. Again, affirming the importance of a good arena set up and reliable methods.

The activity test could in this case gain from movement monitoring through a camera, as this is more precise and a measurement of overall movement instead of changing of zones. An individual would sometimes change directions more often, not necessarily crossing a zone but still move around a lot. Both exposed males and females had a higher mean value when it came to changing zones, which is consistent with previously mentioned results (Bossus et al

2014; De Castro-Català et al 2017). With a more precise measurement, a difference might have been detected. Moreover, fluoxetine's influence on organisms has shown to be quite complex and results tend to contradict each other. Zebrafish (*Danio rerio*) chronically exposed to fluoxetine stayed longer in a freezing position (Maximino et al 2011). At the same time, evidence that fluoxetine can lead to less time spent in a freezing position can be seen in studies on Piaucu fish (*Leporinus macrocephalus*) and Zebrafish (Barbosa et al 2012; Wong et al 2013). Several reasons for this have been suggested. Firstly, as previously discussed, how a particular personality trait is tested might differ between research articles, since they may use different methods to test the same thing. Secondly, fluoxetine might cause effects in low doses that can not be seen in high doses, a so called non-monotonic dose response (Guler & Ford 2010; Lange et al 2006). Thus, low concentrations over a longer period could affect organisms quite differently than more acute exposure.

In conclusion, pharmaceuticals like fluoxetine continue to leak out through wastewater emission points and fluoxetine can be found in a range of different aquatic organism. There is evidence of fluoxetine's non-target effects as discussed above. Yet, how hazardous fluoxetine is to ecosystems is still unclear. The results from the conducted behavioural assays in this study showed that a low dose exposure of fluoxetine, 20 ng/L, 28 days exposure, influenced *A. aquaticus'* boldness. Fluoxetine along with other SSRIs has, across taxa, proven to influence behavioural responses. However, fluoxetine's effect on organisms differ and is dependent on exposure period, dose and how sturdy an organism is towards the substance. Although this study could not see behavioural difference in exploration, activity and fear in fluoxetine exposed *A. aquaticus* does not mean that fluoxetine is harmless. Serotonin is a substance that is important to crustaceans endocrine system (Guler & Ford 2010), and it cannot be ruled out that it affects and disrupts important functions. As mentioned previously, recent studies suggest that pharmaceuticals targeting reuptake proteins can affect invertebrates (Bossus et al 2014; De Castro-Català et al 2017). It is important to remember that pharmaceuticals and their active substances are made to affect physiological processes in our bodies, e.g. receptor bindings, hormone levels, neurotransmitters and more. This is not in any way unique to us humans but shared with many organisms. The serotonergic system is a conserved system and vertebrates and invertebrates share a lot of characteristics and functions (Gillette 2006). Moreover, it has been suggested that the vertebrate serotonergic nervous system might originate from the invertebrates (Hay-schmidt 2000). Due to these close similarities between vertebrates and invertebrates, it is crucial to keep investigating the impact of pharmaceuticals on organisms and ecosystems, to highlight and prevent potential disrupting threats.

5.1. Ethical and Social aspects

Swedish legislation has regulations for animal testing (SFS 2019:66). Permits are only required for mammals, birds, reptiles, fish, cyclostomes and octopus/cuttlefish. Thus, there is no regulation or ethical requirement for using isopods. However, the organisms can still be

stressed when they are treated and handled during this study and should therefore be handled in a correct manor.

The usage of pharmaceuticals has increased with around 60% the past decade, resulting in an increased number of medical residues in our freshwater environments. The main sources are wastewater treatment plant emission points. Pharmaceutical production, laboratories and hospitals are contributing a major part of the overall residuals. As pharmaceuticals are made to influence chemical interaction in the human body, it may also influence other organisms. Pharmaceuticals could possibly alter the normal behaviour in freshwater organisms and in a broader perspective alter ecological interactions.

Sweden has a set of national environmental objectives to protect our environment. Two of these states that Sweden will strive to keep lakes and streams flourishing and keep the overall environment free from toxic substances. This study will contribute to further knowledge about fluoxetine's toxic effects and how this effect organisms inhibiting lakes and streams.

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