Effects of pharmaceuticals on fish behavior

Oxazepam impact on social preferences and responses on predation risk (olfactory cue mixture) in guppies

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Abstract

Effects of oxazepam concerning on social behavior in guppies are still unknown. The purpose of this thesis is to investigate if the benzodiazepine oxazepam has effects on fish behavior in terms of social preferences and responses to predation risk using an olfactory cue mixture. After an exposure period of 15 days to 100 µg/l of oxazepam, behavioral experiments were performed over two days. Results indicate that oxazepam exposed fish were more social at the beginning of the experiment, which differ from what was expected and from previous social preferences studies. Moreover, less social behavior was found as a result of combining oxazepam treatment and olfactory cue mixture (predator cues and guppy skin extract) treatment.

Key words: Oxazepam, Behavior, Predator cues, Guppies.
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1. Introduction

Pharmaceuticals are a potential group of environmental contaminants (Calisto and Esteves, 2009), but this problem did not attract major attention until the 1990s when it was found that some compounds have the capacity to interfere with ecosystems in concentrations as low as few nanograms per liter (Calisto and Esteves, 2009). A huge diversity of pharmaceuticals has been found in the environment: analgesics, antibiotics, anti-epileptics, b-blockers, blood-lipid regulators, antidepressants, anxiolytics, sedatives, contraceptives, etc. (Jones et al, 2006). Oxazepam is a benzodiazepine used to treat anxiety and it has been widely prescribed for humans since the 1960s and nowadays it can be found in aquatic environments (Brodin et al, 2013). The contamination mechanism is simple: patients ingest the pharmaceutical and then, a part of the drug is excreted still biologically active and enters into the aquatic environments through treated wastewater effluents (Calisto and Esteves, 2009). Recent studies have demonstrated that oxazepam cause behavioral modifications in fish (Brodin et al, 2013).

Variability in behavioral traits has important ecological and evolutionary implications (Smith and Blumstein, 2008) especially in responding to changes in environmental conditions such as predation pressure, food availability and social condition. Differences between individuals in aggressiveness, activity levels, sociability and the tendency of taking risk have been documented (Piyapong et al, 2010). Social interactions are also influenced by a number of ecological factors (Croft et al, 2005). Predation pressure has been shown to be a strong environmental factor influencing the behavior of some fishes (Goldenberg et al, 2014), including guppies (James A. Farr, 1975). For example, it has been discovered that sexual behavior and degree of social interactions of guppies vary drastically as a function of type of predation pressure (James A. Farr, 1975).

Additionally, many fishes have been shown to possess chemical alarm signals, localized in the skin. These chemicals are generally only released through mechanical damage to the skin, as would occur during predation (Smith, 1992) and are utilized to warn conspecifics of a predation threat (Brow and Smith, 1998). The alarm pheromone is called schreckstoff and induces a response, characterized by an increase in a variety of anti-predator behaviors, including dashing, freezing, shelter use, shoaling, reduced foraging, and avoidance of areas where alarm signals have been detected (Brow and Smith, 1998). It has been shown the presence of a chemical alarm signal (alarm pheromone) in the guppy, when exposed to a skin extract of conspecifics, and demonstrated that alarm pheromones may function to mediate predation risk under natural conditions in the guppy (Brown and Godin, 1999).

The disturbance of this kind of behavioral responses by pharmaceutical contaminants may affect the individual fish, trophic cascades and aquatic environments (Brodin et al, 2014).

The purpose of this thesis is to investigate if the pharmaceutical oxazepam, has effects on fish behavior in terms of social preferences and responses on predation risk using an olfactory cue mixture. Female guppies were used because it is still unknown if oxazepam affects social behavior or the response to predation risk of this species. This study focuses on answering the following questions: (1) Does oxazepam change the social preferences and the activity of guppies under normal conditions (fresh water)? (2) Does oxazepam affect these two behavioral parameters in the same way when fish experience predation risk (olfactory cue mixture)? (3) Are there temporal changes in social preferences and activity of guppies during the behavioral experiments? (4) Are these temporal changes different in response to predation risk (olfactory cue mixture) or oxazepam exposure? (5) Are fish affected in their behavioral responses by being tested twice?
2. Materials and Methods

2.1 Exposure period

The 7th of April 2015 a number of 32 female guppies (*Poecilia reticulata*) from river Turure in Trinidad, bred in laboratories from Umeå Universitet (Sweden) were collected and individually placed in aquaria (13 x 12.5 cm and 21 cm high, filled with 2 liters of aged tap water) (fig. 1). The fish had visual contact between each other to reduce stress, and were daily fed with a diet of commercially prepared flake food. Aquaria were aerated and covered by perforated plastic to prevent the fish from jumping out of the aquaria (fig. 1). Every second day water levels were controlled in all aquaria and fresh water was used to refill the evaporated water. Illumination was provided by overhead fluorescent strips and the water temperature was maintained at 24 ± 1°C. Half of the fish were exposed to 100 µg/l of the anxiolytic drug oxazepam, and the other half were kept in fresh water without pharmaceuticals as control samples. The exposure period lasted 15 days. Water samples were taken twice, at the beginning of the experiment (8.04.2015) and at the end (23.04.2015). When the study was finished, fish were killed with an overdose of tricaine methanesulfonate (MS222) and stored in the freezer for later analysis. However, the chemical analysis for oxazepam concentrations in water and fish will not be part of this thesis.

The number of times the fish ate was noted during 5 days (10, 11, 12, 13 and 14 of April) for later analysis following the described procedure for each individual fish: the food was put into the aquaria, the observer (EC) waited 10 seconds two steps away from the aquaria (to avoid stressing the fish) and it was noted if the fish ate during this period of time.

The experiments were permitted by the Ethical Committee on Animal Experiments in Umeå (license Dnr: A19-15).

2.2 Skin extract elaboration

Skin extract was used as chemical alarm cue to simulate the presence of a predator and obtain responses to predation risk from the fish. The skin extract was elaborated following the described procedure from Brow and Smith (1998). Thirty-one male and female guppies were taken from the laboratory and were sacrificed by decapitation. Tails and entrails were removed, only the skins were used. In total 29.7 cm³ of skin was homogenized with a final concentration of 300 ml distilled water. The machine used in the procedure is shown below (fig. 2). The preparation was stored in the freezer until the day of the experiment.
2.3 Behavioral Experiments

The experiment was performed over two days starting the 22.04.2015. All fish (n=25) were tested twice, once on day 1 and again on day 2. Two big tanks (60 x 30 cm x 35 cm high) were used. Also two transparent partitions divided each tank transversely, so that in the end, each big aquarium was divided in three areas. The two outer compartments were each 15 cm long x 30 cm high x 35 cm wide and the middle compartment was 30 cm long x 30 cm high x 35 cm wide (fig. 3). Both tanks were filled with 11 liters of fresh water. But the middle area of one tank included 5 ml of guppy skin extract and 162.5 ml of cichlid water from *Rocio octofasciata* (predator cues). The used concentrations were higher than Brow and Smith (1998) study to be sure to get an effect and the term “olfactory cue mixture” in this study will refer to the mixture of the two substances (guppy skin extract and predator cues). Fourteen female guppies were taken from laboratories and used to represent the two different social groups. They were grouped in the outer areas of the aquariums divided as “big group” (n=5) and "small group" (n=2). The experimental fish were tested in the middle zone of the tanks. Table 1 collects a summary of the 25 fish used throughout the two days of experiment, clearly differentiating the two treatments.

<table>
<thead>
<tr>
<th>Experiment Day</th>
<th>NO</th>
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<tr>
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<td>FW</td>
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<td>1</td>
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<tr>
<td>2</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

2.3.1 Day 1

25 fish were tested on the first day, 12 of them with fresh water and the others 13 with the olfactory cue mixture. As table 1 shows, 10 of the fish were exposed to oxazepam and 15 were not oxazepam treated. The fourteen fish grouped in the outers areas of the aquariums (representing the two different social groups) were placed one hour before into the tank in order to acclimate. They were switched when half of the experimental fish were already tested to avoid positional preferences. After being switched, one hour more was allowed before testing the remaining fish. Testing was carried out by introducing the experimental fish into the middle area of the aquarium and by videotaping for the following 20 minutes, for subsequent analysis.

2.3.2 Day 2

The same setup was used on day 2. The 25 fish were tested but this time 12 fish (mixed unexposed and oxazepam exposed) were examined with the same treatment as day 1. Therefore, 5 of them were analyzed with fresh water and 7 with the olfactory cue mixture. The remaining 13 fish were tested with the opposite treatment than day 1. The same fourteen female guppies represented the two social preferences in the same way and every experimental fish was videotaped following the identical procedure as Day 1.

2.4 Video Analysis

Videos were analyzed using Perchmon program. The area of analysis was divided in five identical zones for observation (fig. 3). Zone 0 was always placed close to the small group of guppies and zone 4 was placed close to the big group of fish (fig. 3). The program was used in order to record the time spent by the fish in each zone along the first 16 minutes, although the recording time lasted 20 minutes, as stated above. The video data was used to calculate the following variables:
1) The time during 0-8 minutes that the fish spent in each zone.

2) The activity (number of crosses between the five zones) during 0-8 minutes.

3) The time during 8-16 minutes that the fish spent in each zone.

4) The activity (number of crosses between the five zones) during 8-16 minutes.

5) The total time that the fish spent in each zone over the entire 16 minutes.

6) The total activity (number of crosses between the five zones) over the entire 16 minutes.

7) Temporal differences in activity, time spent in V0 and time spent in V4 between the two periods of time (0-8 min/8-16 min). Calculated as follows:
   - Temporal differences in activity = [Number of total crosses between the five zones during 8-16 min]–[Number of total crosses between the five zones during 0-8 min]
   - Temporal differences in time spent in V0 = [Time in V0 during 8-16 min]-[Time in V0 during 0-8 min]
   - Temporal differences in time spent in V4 = [Time in V4 during 8-16 min]-[Time in V4 during 0-8 min]

8) Two-days differences in activity, time spent in V0 and time spent in V4 between experimental day 1 and day 2. Calculated as follows:
   - Two-days differences in activity = [Number of total crosses between the five zones in day 2]–[Number of total crosses between the five zones in day 1]
   - Two-days differences in time spent in V0 = [Total time in V0 in day 2]-[Total time in V0 in day 1]
   - Two-days differences in time spent in V4 = [Total time in V4 in day 2]-[Total time in V4 in day 1]

9) Combined-treatments differences in activity, time spent in V0 and time spent in V4 between olfactory cue mixture treatments (FW=Freshwater; P=Predation risk with olfactory cue mixture) during behavioral test. Calculated as follows, for unexposed and oxazepam exposed fish (exposure treatment) separately:
   - Treatment differences in activity = [Number of total crosses between the five zones with FW]–[Number of total crosses between the five zones with P]
   - Treatment differences in time spent in V0 = [Total time in V0 with FW]-[Total time in V0 with P]
   - Treatment differences in time spent in V4 = [Total time in V4 with FW]-[Total time in V4 with P]

Figure 3. Experimental design structure. Observed zones marked with dashed lines. On the left side, the small fish group (n=2), on the middle, the fish tested and on the right side, the big fish group (n=5).

2.5 Statistical Analysis

The data was sorted in Excel (version 2013) before exporting to SPSS for visual interpretations and statistical testing. Normality of the data was tested with Shapiro-Wilk tests and homogeneity of variances with Levene’s tests.
To analyse the differences between no oxazepam exposed (NO) and oxazepam exposed (OX) fish for feeding during the exposure time, a Mann-Whitney test was used because data was not normally distributed. To analyse the time spent in each zone (V0; V1; V2; V3; V4) and total activity between no oxazepam exposed (NO) and oxazepam exposed (OX) fish, and conditions of freshwater (FW) and predation risk with olfactory cue mixture (P) during the behavioral experiments for day 1 a two-way Analysis of Variance (ANOVA) was used. A two-way ANOVA was also used to test behavioral differences between days and the differences in time during the behavioral experiments between NO/OX and FW/P treatments. A T-test was used to analyze differences in activity, time in V0 and time in V4 between NO/OX and FW/P. When data was not normally distributed it was log transformed to meet the requirements for parametric tests. Statistical significance was defined as a p-value of <0.05.

3. Results

In total, 21.88% of the fish died (i.e. 7 of 32) during the exposure period, one unexposed and six oxazepam exposed fish.

3.1 Feeding

Feeding was estimated by giving numbers to all fish depending on whether the fish ate during the procedure previously described. Over five days of feeding observation, a “0” was assigned if fish did not eat and “1” if they did. At the end, numbers assigned were added and they revealed the frequency of the feeding. Thereby, final numbers with their meanings given were as follow: 0=never ate; 1=rarely ate; 2 and 3=sometimes ate; 4=almost always ate; 5=always ate (fig. 4). No significant differences (Mann-Whitney Test: p=0.441) were found in feeding between oxazepam exposed (OX) and no oxazepam exposed (NO) fish. Nonetheless, data show that fish treated with oxazepam had a double frequency value compared to unexposed fish (fig. 4).

![Figure 4. Feeding frequency (using the following nomenclature: 0=never ate; 1=rarely ate; 2 and 3=sometimes ate; 4=almost always ate; 5=always ate) of guppies during the exposure period (NO=No Oxazepam; OX=Oxazepam) over five days of feeding observation. Error bars represent ±1 SE (n=26).](image)

3.2 Behavioral experiments
3.2.1 Day 1

In day 1, no oxazepam exposed (NO) and oxazepam exposed (OX) fish tested in fresh water (FW) and fish tested with predation risk with the olfactory cue mixture (P) did not show a clear preference for any of the five areas, and there was no significant interaction effects between both treatments (ANOVA: Total time in V0 (I)NO/OX, p=0.454 (II)FW/P, p=0.414 (III)Interaction, p=0.298; Total time in V1 (I)NO/OX, p=0.959 (II)FW/P, p=0.381(III)Interaction, p=0.075; Total time in V2 (I)NO/OX, p=0.578 (II)FW/P, p=0.562 (III)Interaction, p=0.471; Total time in V3 (I)NO/OX, p=0.116 (II)FW/P, p=0.904 (III)Interaction, p=0.833; Total time in V4 (I)NO/OX, p=0.591 (II)FW/P, p=0.101 (III)Interaction, p=0.311). There was also no significant effect of the two treatments on the activity for data from day 1 (ANOVA: Activity (I)NO/OX, p=0.961 (II)FW/P, p=0.523 (III)Interaction, p=0.136). Overall, guppies seem to be very social, because they prefer to spend more time close to both groups of fish (V0 and V4) than in the middle of the aquarium, regardless of group size (fig. 5). This tendency is notable in fish tested with fresh water and with the olfactory cue mixture and is not influenced by any of the treatments (see statistics above).

Figure 5. Averages of total time (seconds) spent in V0, V1, V2, V3, V4 and activity (number of crosses between the five zones), combining both treatments (exposure treatment: NO=No Oxazepam; OX=Oxazepam; and olfactory cue mixture treatment: FW=Freshwater; P=Predation risk with olfactory cue mixture), of fish tested in day 1 (n=25). Error bars represent ±1 SE.
Positive values of temporal differences in time spent in V0 or time spent in V4 mean that guppies spent more time in these zones between 8-16 minutes and on the other hand, negative values mean guppies spent more time in these zones between 0-8 minutes (fig. 6). No significant effects of oxazepam treatment and olfactory cue mixture treatment were found for time spent close to the small group (V0) and activity along the time of the experiment (ANOVA: Temporal differences in time spent in V0 (I) OX/NO, p = 0.234 (II) FW/P, p = 0.362 (III) Interaction, p = 0.728; Temporal differences in activity (I) OX/NO, p = 0.348 (II) FW/P, p = 0.413 (III) Interaction, p = 0.639). However, the effect of oxazepam treatment on time spent by fish in V4 (close to the big group of fish), was close to significance (ANOVA: Temporal differences in time spent in V4 (I) OX/NO, p = 0.051 (II) FW/P, p = 0.419 (III) Interaction, p = 0.655), which could indicate that oxazepam exposed fish prefer to spend more time close to the big group of fish during the first half (0-8 min) of the behavioral experiment.

Figure 6. Temporal differences between first period (0-8 min) and second period (8-16 min) of the experiment. Temporal differences in activity (= [Number of total crosses between the five zones during 8-16 min]–[Number of total crosses between the five zones during 0-8 min]), temporal differences in time spent in V0 (= [Time in V0 during 8-16 min]–[Time in V0 during 0-8 min]) and temporal differences in time spent in V4 (= [Time in V4 during 8-16 min]–[Time in V4 during 0-8 min]) combining both treatments (exposure treatment: NO=No Oxazepam; OX=Oxazepam; and olfactory cue mixture treatment: FW=Freshwater; P=Predation risk with olfactory cue mixture), of fish tested on Day 1 (n=25). Error bars represent ±1 SE.
3.2.2 Day 2

Results from day 2 display differences from guppies tested with the same treatment in day 1 and day 2 (fig.7). Positive values of two-days differences in time spent in V0 or time spent in V4 mean that guppies spent more time in these zones on day 2 and on the other hand, negative values mean guppies spent more time in these zones on day 1 (fig.7). No significant effects of oxazepam treatment and olfactory cue mixture treatment were found for two-days differences on social preferences and activity between the two days of experiment (ANOVA: Two-days differences in time spent in V0 (I)OX/NO, p=0.096 (II)FW/P, p=0.389 (III)Interaction, p=0.829; Two-days differences in time spent in V4 (I)OX/NO, p=0.792 (II)FW/P, p=0.958 (III)Interaction, p=0.814; Two-days differences in activity (I) OX/NO, p=0.952 (II)FW/P, p=0.168 (III)Interaction, p=0.765).

Figure 7. Two-days differences between experimental day 1 and day 2. Two-days differences in activity (=Number of total crosses between the five zones in day 2– Number of total crosses between the five zones in day 1), two-days differences in time spent in V0 (=Total time in V0 in day 2-Total time in V0 in day 1) and two-days differences in time spent in V4 (=Total time in V4 in day 2-Total time in V4 in day 1) of fish under oxazepam treatment (NO=No Oxazepam; OX=Oxazepam) tested both days with the same treatment during the behavioral test (olfactory cue mixture treatment: FW=Freshwater or P=Predation risk with olfactory cue mixture) (n=12). Error bars represent ±1 SE.
Positive values of treatment differences in time spent in V0 or time spent in V4 mean guppies (separately, no oxazepam exposed (NO) and oxazepam exposed (OX)) spent more time in these zones if they were treated with fresh water and on the other hand, negative values mean fish spent more time in these zones if they were treated with the olfactory cue mixture (fig. 8). No significant effects of combined-treatments differences on social preferences for time spent close to the big group (V4) and activity were found between oxazepam exposed and no oxazepam exposed fish (T-Test: Treatment differences in time spent in V4, p=0.153; Treatment differences in activity, p=0.184). However, oxazepam effect under predator pressure related with the time that fish spent in V0 (close to the small group), was close to significance (T-test: Treatment differences in time spent in V0, p=0.094), which could indicate that oxazepam exposed fish prefer to spend more time close to the small group of fish if they were treated with fresh water and control fish seems to prefer to spend more time close to the small group of fish if they were treated with the olfactory cue mixture (fig. 8 and fig. 4).

Figure 8. Differences of exposure treatment (NO=No Oxazepam; OX=Oxazepam) separately, combined with treatment during behavioral test (olfactory cue mixture treatment: FW=Freshwater; P=Predation risk with olfactory cue mixture) in activity (=Number of total crosses between the five zones with FW–Total activity with P), time spent in V0 ([Total time in V0 with FW] – [Total time in V0 with P]) and time spent in V4 (=Total time in V4 with FW–Total time in V4 with P) of fish tested both days (n=13). Error bars represent ±1 SE.
4. Discussion

4.1 Feeding

According to Brodin et al. (2013) fish exposed to the anxiolytic drug oxazepam feed earlier and deplete the food resource faster than those unexposed fish. Research from Brodin et al. (2013) was performed on wild European perch (\textit{Perca fluviatilis}). The same result was expected for guppies but there was no significant difference in feeding frequency between oxazepam exposed and no oxazepam exposed guppies (fig. 4). This might be due to the experimental procedure used in this study and the very short observation time (10 seconds duration), which might have not been enough to see a strong effect. It is probable that fish ate after this short period of time but it was not noted during the procedure. Feeding rates have been tested in other studies with higher observation times. For example, Brodin et al. (2013) found that juvenile perch exposed to 1.8 µg/L of oxazepam only started eating after 25 second. Furthermore, the exposure pharmaceutical concentrations could also interfere with the results, being more probable to find differences if fish are exposed to a higher amount of drug. Therefore, the exposure concentration used in this study might have been too low to affect guppies. In surface waters, concentrations usually range from low ng/L to low µg/L, but certain point sources, such as pharmaceutical production and manufacturing facilities, can result in concentrations as high as mg/L in receiving surface waters (Brodin et al, 2014).

As previous studies have reported, oxazepam effect on feeding can result in an accelerated exhaustion of the food resource and therefore, aquatic ecosystems could be affected. These ecological consequences include potential effects on individual’s survival and reproductive success, population dynamics (e.g. growth, fecundity, and survival), community structure and species diversity (through influences on species interactions), and effects on the conservation and management of natural resources (Mittelbach et al, 2014).

4.2 Behavioral experiments

Previously published studies have demonstrated that oxazepam has significant effect on fish behavior, increasing activity and decreasing sociability (Brodin et al, 2013). However, behavioral effects of pharmaceuticals can differ between species and they are also defined by the amount of drug taken up by the fish (Brodin et al, 2014). This study shows no effect in relation with activity, but oxazepam exposed fish tend to spend more time next to the small group of fish (fig. 5) which could be interpreted as a low level of sociability and therefore, be in agreement with findings from earlier studies. Effects of pharmaceuticals on behavior are of direct ecological importance, as behavior is tightly linked to individual fitness and population persistence (Smith and Blumstein, 2008). Reduced sociality results in lower prevalence of shoaling and may also increase predation risk (Brodin et al, 2013). Therefore, these changes in individual fitness may also produce indirect ecological effects, such as predation or competition (Brodin et al, 2014), resulting in population increase, decrease, or even local extinction (Werner and Peacor, 2003).

In most animal species, predator avoidance is crucial, and individuals often adjust their behavior in accordance with perceived predation risk (Brodin et al, 2014). Predation has long been implicated as a major selective force in the evolution of several morphological and behavioral characteristics of animals (Lima and Dill, 1990). Typically, predator avoidance involves reduced activity to minimize encounter rates with potential predators, but an activity reduction often means less feeding and growth and, hence, reduced fitness. Both control fish and oxazepam exposed fish under predation risk situation from this study do not display decreasing in activity. However, the responses of the two groups are a little different. While control fish tend to increase activity under predator pressure, fish exposed tend to decrease activity in these same conditions, but in any case not significantly in this study (fig. 5). Moreover, not patent changes on social preferences were found comparing exposed and unexposed fish (fig. 5). Nevertheless, it should be taken into account that the physical and cognitive capacity of domesticated guppies may differ from wild guppies (Burns et al, 2009) and fish used in this study have never experienced conditions of predation risk before, which could have determined the behavior observed.
Nevertheless, there was a strong tendency of guppies to avoid central zones of the aquarium, indicating that fish normally prefer the company of other fish, no matter the size of the shoal. It is known that guppies swim in shoals for social and protectoral reasons (Magurran and Seghers, 1994).

This study has shown a change in social preferences suggesting that fish under oxazepam conditions seem to be more social in a first contact to a new environment. A previous study has shown that the pharmaceutical oxazepam increases boldness and decreases sociability in fish (Brodin et al, 2013), but the development or progression of this social condition has never been tested. A trend from oxazepam exposed guppies to swim with the small group of fish during the experiment was expected, but at the beginning they showed higher preference on swimming with the big group of fish, indicating high sociability. This tendency demonstrates that fish behavior under oxazepam effects is not constant, and it develops and changes during the time.

Moreover, these temporal differences on social preferences in guppies might be affected by the presence of predation risk because evidences of potential interaction with oxazepam were found in this study (fig. 8). Gong and Gibson (1996) suggest that predators can influence social and sexual preferences in female guppies. Goldenberg et al. (2014) demonstrated that with increasing predation risk, juvenile perch (Perca fluviatilis) reduce boldness and intensified vigilance. In this study when unexposed fish have been tested with the two treatments (fresh water and predator pressure conditions), one each different experimental day, lower sociability is shown under predation risk (fig. 8), in agreement with James A. Farr (1975) who found that guppies lost the tendency to aggregate under predation pressure influencing social behavior patterns. However, contrary to these findings, this study indicate that oxazepam exposed fish change this preference when they are tested also with the two treatments (fresh water and predator pressure conditions) in the same way. They seem to be more social with the presence of the olfactory cue mixture (fig. 8), indicating how this pharmaceutical alters the natural behavior of fish. The alteration of responses to predation could have serious ecological consequences, taking into account that predation has been claimed to be one environmental parameter which is a major influence on the biology of the guppy (James A. Farr, 1975).

Additionally, the repetition of testing during two days did not present significant consequences in this research. Repeated measures may be essential to correctly interpret certain relationships in behavior (Goldenberg et al, 2014). Previous investigations have found individuals’ boldness is influenced by the repeated measure as well as a more predictable reaction towards the predator (Goldenberg et al, 2014). This is a result of progress in habituation to the new environment. Moreover, habituation has a great potential to affect patterns in response to group size and predation risk and should ideally be considered through repeated measures when studying behavior (Goldenberg et al, 2014).

Finally, this study complements other researches that have included the full pharmaceutical–behavioral–ecological sequence of potential impacts (Brodin et al, 2014) and results become difficult to interpret, synthesize, and extrapolate, given that aquatic wildlife living in contaminated environments is exposed to a wide range of pharmaceuticals that could lead to additive or non-additive effects or even neutralize each other’s effects (Backhaus, 2014). Therefore, there is a need for further researches on how drugs affect the behavior of fish and to what extent. It should be noted that this study has been conducted in a laboratory under simplified and controlled conditions. Studies of behavioral trait variation under natural or semi-natural conditions are still quite unusual (Mittelbach et al, 2014). Loos et al (2013) measured oxazepam concentrations in treated effluent water up to 1.9 μg/L. Rivers and streams benzodiazepines range from 0.001 to 0.4 /L (Hummel et al, 2006). Focusing on Swedish environment, surface waters have shown oxazepam concentrations of 0.73 mg/L in treated wastewater effluent and 0.58 mg/L in a mid-sized stream (Brodin et al, 2013). Although the concentration used in this study (100 μg/L) was far above concentrations found in the environment, a previous research has found effects of oxazepam causing changes in behavior under the same values as in this study (Swärd, 2015). Furthermore, oxazepam concentrations in guppies from this study were not measured, so further comparisons are not possible.
In conclusion, I found a trend on guppies to change behavior when they are exposed to the pharmaceutical oxazepam in terms on social preferences and responses to predation risk. However, future studies should investigate how accurate behavior is studied under controlled laboratory conditions regarding the biotic (e.g. interaction with other organisms) and abiotic factors (e.g. natural changes in temperature and pH) occurring in the real natural environment of the organism. The study highlights the difficulty to simulate complex ecological relationships and natural behaviors in a small sample under artificial circumstances. Complementary analysis are needed for better understanding as well as to verify the findings of this study.

5. Acknowledgement

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6. References


