How Pharmaceutical Pollution Affects Social Behavior in a Cichlid Fish

Influence of diazepam on social attraction and group spacing in *Neolamprologus multifasciatus*

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Swedish University of Agricultural Sciences, SLU
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Abstract

Pharmaceutical pollution is an emerging threat to aquatic ecosystems, yet its impact on complex social behaviours in fish remains underexplored. This thesis investigates how diazepam, a commonly detected Anxiolytic drug affects group spacing and social attraction in the shell-dwelling cichlid *Neolamprologus multifasciatus*. In a controlled laboratory setting, fish were exposed to three treatment levels of diazepam, control (0 µg/L), low (2 µg/L), and high (50 µg/L). Group spacing was assessed by analysing the spatial clustering of shell shelters used by the fish, while social attraction was evaluated through a two-choice behavioural task in a separate testing aquarium. Results showed that fish exposed to high doses of diazepam formed less clustered territories, indicating reduced group cohesion. In the two-choice sociality trials, high dose fish emerged more quickly from their shell shelters, spent more time near conspecifics, and exhibited increased exploratory behaviour. These changes suggest enhanced social attraction and boldness, consistent with diazepam's known anxiolytic effects. The observed effects were dose-dependent, with stronger behavioural changes at higher concentrations. The effect on social attraction were stronger in females, who showed stronger behavioural changes than males. These findings demonstrate that diazepam can alter both spatial and social behavioral aspects of a group living social fish species. Such behavioural disruptions show the need to consider complex social behaviours when evaluating the environmental impact of pharmaceutical contaminants.

Keywords: Behaviour ecology, Ecotoxicology, Pharmaceutical pollution, Anxiolytic drug, Diazepam, Cichlid Fish, *Neolamprologus multifasciatus*, Social attraction, Territoriality, and Group spacing

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1. Introduction

Pharmaceutical pollution is an increasing global environmental issue, with anxiolytic substances such as diazepam frequently detected in our waterways (Brodin et al. 2014; Wilkinson et al. 2022). These substances enter the environment primarily through wastewater effluents, following human production and consumption (Wilkinson et al. 2022). These pollutants are considered pseudo-persistent in the water column, where they over time gradually degrade, but are at the same time continuously replenished by wastewater effluents, allowing them to remain biologically active in the environment (Klaminder et al. 2015; Wilkinson et al. 2022). Aquatic organisms can be exposed to these contaminants via surface water, where concentrations of 20-50 ng/L diazepam have been noted (Wilkinson et al. 2022). Diazepam, also called Valium^{TM}, is a benzodiazepine primarily used as an anxiolytic drug to manage anxiety disorders in humans (Pritchett et al. 1989). It alters human behaviour by enhancing the effects of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter that reduces neuronal excitability, leading to a calming effect on the central nervous system (Calcaterra & Barrow 2014; Pritchett et al. 1989). Because many organisms, including fish, possess similar receptors as humans, diazepam is thought to affect them in comparable ways (Carr & Chambers 2001).

Despite the increasing pharmaceutical pollution problem, most ecotoxicology studies in fish focus on simplistic endpoints such as swimming activity, while more complex behaviours like social attraction and grouping remain less explored. Previous studies have often tested fish in social isolation, even though many species (particularly social fish) live in groups and depend on interactions with other individuals for key life processes like predator evasion, foraging, or mating (Martin & McCallum 2021). Although research has shown the impact pharmaceuticals like anxiolytics have on fish behaviour, it remains important to understand how such substances affect social grouping behaviours (Michelangeli et al. 2022). Sociality refers to the tendency that individuals of a species have to form groups and is a key behavioural trait in many animals (Ward & Webster 2016). Group living can provide numerous benefits, including improved access to resources and better protection from predators (Ward & Webster 2016). One fundamental aspect of sociality is social attraction, which describes the tendency of individuals to seek spatial proximity with other individuals (Ward et al. 2020). Social attraction forms the foundation for group formation, as it describes the ability to recognise other individuals and the motivation to initiate and maintain social interactions with others (Ward et al. 2020).

Given the ecological importance of grouping behaviours in many animals, understanding how pharmaceuticals affect these behaviours is crucial for assessing the broader environmental impact this kind of pollution has on ecosystems (Ward & Webster 2016). A growing body of research shows that pharmaceutical pollutants, including anxiolytics, can significantly alter fish behaviour (Brodin et al. 2014). Such behavioural changes may have far reaching effects, as many fish species rely on their ability to live in groups for essential functions like territory defence, reproduction, and predator avoidance (Brodin et al. 2014; Jordan et al. 2016). Previous research on the effects of benzodiazepine exposures, include reduced aggression and activity, increased boldness and changed social interactions and attraction (Brand et al. 2025; Brodin et al. 2013; Brodin et al. 2014; Jordan et al. 2016; McCallum et al. 2021). For example, Brand et al. (2025) found that Atlantic salmon (Salmo salar) exposed to the benzodiazepine, clobazam, showed increased risk-taking, along with disrupted grouping and social cohesion. These findings raise concerns about how anxiolytic drugs may interfere with the ability of exposed fish to form and live in social groups.

In my thesis, I evaluated how the anxiolytic medication diazepam affects social attraction and grouping of fish, using a social cichlid as a model organism. *Neolamprologus multifasciatus* is a small, highly social group living cichlid, endemic to Lake Tanganyika in east central Africa (Jordan et al. 2016). The fish territories containing social groups typically consisting of multiple breeding individuals, one to three males (one dominant breeding male) and up to five breeding females and their juveniles (Bose et.al 2022). Fish in these groups establish a social hierarchy and interact regularly with their group members and close neighbours (Bose et al. 2021; Jordan et al. 2016).

The cichlid is a shell dweller meaning it uses empty gastropod shells from the freshwater snail *Neothauma tanganyicense* for shelter and breeding chambers (Bose et al. 2021). These shells are excavated from the sand using the fish's mouth and tail resulting in clusters of shells and sand ridges that form distinct group territories (Lein & Jordan 2021; Salzburger et al. 2014). Each fish resides within one empty shell, so the spatial arrangement of shells within a territory also determines the spatial arrangement of group members. Territory defence is primarily carried out by the dominant male, often through male to male aggression (Bose et al. 2022). Females defend their own sub territories (within the group's broader territory), typically against other females (Bose et al. 2022; Jordan et al. 2016). Like many other social animals, *N. multifasciatus* depend on grouping behaviours for survival and reproduction making them an ideal model species to study how pharmaceutical pollutants may disrupt social attraction and group spacing (Bose et al. 2022).

The aim of this thesis was to look at the dose-dependent effects of diazepam exposure on social grouping behaviour of *N. multifasciatus*. My thesis asked whether diazepam alters social attraction and grouping among exposed fish. Given that benzodiazepines have been shown to reduce anxiety (one reason why fish may form groups in the wild) and social cohesion (Carr & Chambers 2001; Ward & Webster 2016), I predicted that diazepam will cause reduced group cohesion, reflected as less spatial clustering of the fish's shell shelters. Further, because benzodiazepines have been shown to increase disinhibition (Brodin et al. 2014; Brand et al. 2025; McCallum et al. 2021), I predicted an increase in social attraction, measured as increased time spent in proximity to other *N. multifasciatus* in a separate behavioural assay arena. I predicted that there will be a dose-dependent effect, where there will be a stronger effect at higher concentrations of diazepam. I also predicted that there will be a higher effect of diazepam on females than males, due to biological sex differences (Michelangeli et al. 2022; Wilson et al. 2004).

To test these predictions, these following research questions were answered:

1. Does spatial arrangement of shells placement, group spacing and *N. multifasciatus* territories change in response to diazepam exposure and is this effect dose-dependent?

2. How does exposure to diazepam contaminated water affect social attraction exhibited by *N. multifasciatus*? Is this effect dose-dependent? Is there a difference between sexes?

2. Materials and Methods

2.1 Experimental setup and exposure

N. multifasciatus is well suited for studying the effects of diazepam on social behaviour due to their ability to form distinct social groups in which individuals live in close proximity to one another (Bose et al. 2022). To ensure a genetic mixture the adult *N. multifasciatus* were purchased from several different fish suppliers, including Alex Tropicals (Czech Republic), Zoo.se (Sweden), Hageby Cikliden (Sweden) and Aquarium Glaser GmbH (Germany). The fish were intermixed and kept in the laboratory at SLU, Umeå, in three 400 litre stock tanks, each equipped with airstones, a recirculating biological filter, aquarium sand, *N. tanganyicense* shells and aquarium plants. This study started 23 January 2025 (Day 1) when diazepam was added to the treatment tanks. Four females, and three males marked with elastomer tags (to facilitate individual identification over the study period) were placed in 21 treatment tanks together with 24 *N. tanganyicense* shells, placed in a grid like pattern on a 5cm layer of cichlid sand (Aquadeco Ciklidsand). Each treatment tank was equipped with a recirculating biological filter (Aquaclear 70, with no carbon insert), aquarium heater (Eheim Thermocontrol 100 W) and an air stone.

The fish have been exposed to different levels of diazepam (Diazedor, Salfarm Scandinavia – an aqueous solution of diazepam in ethanol). There were 21 treatment tanks in total (50x70x40 cm, approx. 115L). These tanks were divided into three treatments groups, control, low and high, where there was seven of each. Treatment tanks were exposed by adding the desired exposure concentrations of diazepam dissolved in ethanol into the tank water. Control tanks did not receive any diazepam, but received the same volume of ethanol as the treatment tanks. In the low treatment tanks, a concentration of 2 µg diazepam per litre was aimed for, and in high treatment tanks 50 µg diazepam per litre water was aimed for. The low treatment was chosen as it reflects the high end of what fish may encounter in the wild in a wastewater receiving environment, where a median of 20-50 ngL was found in surface water and extreme cases up to ~1µgL in wastewater effluents in Europe (Fick et al. 2017; Wilkinson et al. 2022). The high treatment was selected as a positive control, it was well below the lethal concentration for fish (LC50, rainbow trout, 84 mg/L) (Straub 2008). Water samples were collected for measuring the concentration diazepam present in the treatment tank water. This thesis did not include the preparation and analysis of these samples, but in short, 12 mL of water was collected, and 5 mL was filtered through a 45 µm filter. It was then analysed using a liquid chromatography mass spectrometry. The average concentration of diazepam in the low treatment tanks

was 1.37 ± 0.43 uμ/L (n = 8 samples), while the concentration in high treatment was 37.68 ± 1.78 uμ/L (N = 3 samples). Note, that only a small subset of the samples was analysed for this thesis, and more samples will be analysed in the future.

To ensure stable water quality, a 25 % water change was carried out every four weeks. The tanks were then re-dosed with new diazepam. The volume of new water (25 % tank volume) was given a full exposure concentration (i.e., 2 uμ/L or 50 uμ/L), while the remaining 75 % of the water was re-dosed at 30 % concentration. This was done to account for degradation of the diazepam across the four weeks. This 30 % re-dosing level was taken from a prior stability analysis (not part of this thesis). The fish were fed with Dr. Basseeler BioFish food (Aquarium Münster), and health and mortality were monitored daily. Water quality parameters: temperature, dissolved oxygen (DO), nitrate, nitrite, hardness (GH and KH), pH and chlorine (Cl) were recorded through test strips (Pro JBL Aquatest Easy 7 in 1), optical dissolved oxygen meter (YSI Ecosense ODO200) and digital pH meter (Hach Pocket Pro+ multi tester). This was carried out every other week and any day mortality were observed, to ensure water quality was stable. The water quality was quite even across the tanks (Appendix 1, Table A).

2.2 Behaviour measures

The goal of the experiment was to assess whether diazepam affects social attraction and group spacing. This was done by doing two main behavioural methods. First, I looked at territorial clustering (modularity). I also assessed individual social attraction by using a two-choice sociality task.

2.2.1 Shell clustering and modularity

Shell spatial clustering and modularity were measured from still frames extracted from top-down videos. Top-down videos of all the 21 tanks were recorded as part of the main study (videos not further used in my thesis) using GoPro Hero 10 black in 4k resolution at 30 fps, with a linear field of view. The recordings used were taken on day 6, 9, 13, 16 and 20 of the study. From still frames taken on each of these days, I extracted the X and Y coordinates of each shell shelter within the exposure tanks.

2.2.2 Sociality

I used a two-choice sociality task to measure fish social preference. In this task, focal fish had a choice of affiliating with two conspecific fish (a male and a female, acting as stimulus fish, each with their own shell shelter) or an asocial side with a compartment that contained no conspecifics and only two shells. Six test tanks (50x27x32 cm; Figure 1) were used, two for low treatment exposed fish,

two for high treatment exposed fish and two for the fish from control groups. Each test tank was divided into three compartments using two transparent glass dividers placed 10 cm from either end wall creating two end compartments (Figure 1). In one of the end compartments, two stimulus fish, one male and one female of a similar size to the focal fish (Appendix 1, Table B) were added from the laboratory stock population tanks together with their two shells. On the opposite end, two empty shells were placed. The shells used were all roughly the same size as the ones used for the exposure tanks. In the middle compartment (30 cm wide), two empty shells were placed adjacent to each glass divider. I added these two simulate a vacant shelter in two groups on opposite sides of the tank, that could be occupied by the focal fish. A focal fish along with its home shell from the exposure tanks, was picked haphazardly from an exposure tank and carefully placed in the middle compartment, 25 cm from the test tank wall (Figure 1). Since all fish start their trials in their shells (as they hide in their shells during transfer between tanks), I was able to quantify time to emerge from their home shell in each of these sociality trials.

In between trials 75 % water was exchanged, and sand and dividers were reset. The stimulus pairs were kept overnight in the test tanks. Sociality trials began on day 43 and continued over seven consecutive days (days 43-49). The stimulus fish pairs switched after the filming on day 46, where two of the old pairs were reused in new test tanks and four new pairs were added (Appendix 1, Table B).

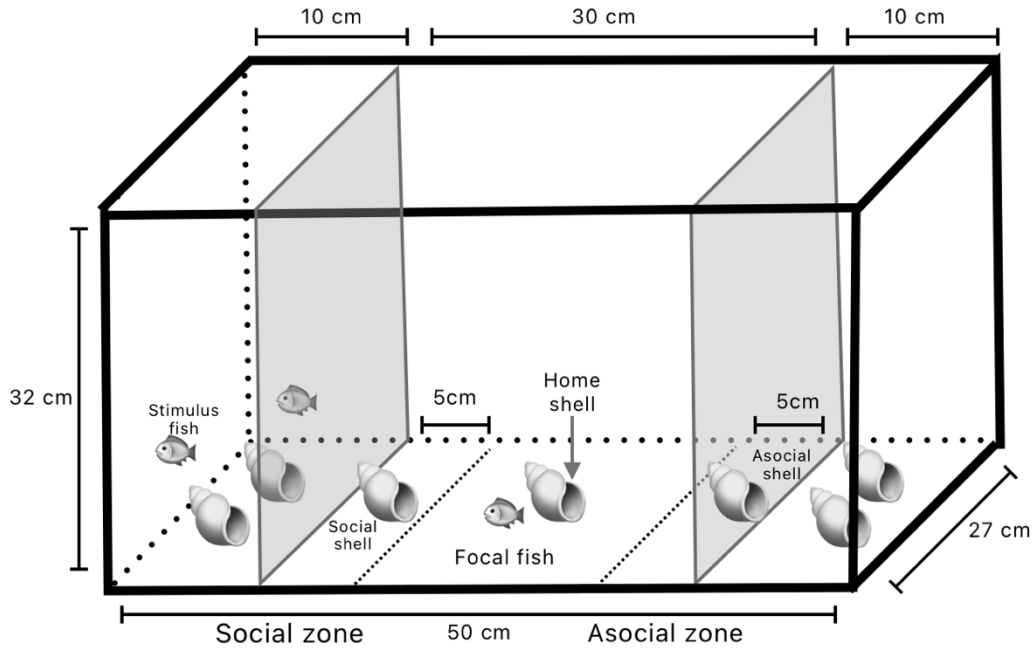

Figure 1. A schematic illustration showing the test tanks used in sociality trails. The dashed lines show the social and asocial zone.

All trials were recorded top down using a GoPro Hero 10 black in 4k resolution at 30 fps, with a linear field of view. Filming lasted approximately 45 minutes per trial, with four tanks recorded at a time, three times a day. The final sample size was: 13 males each from control, low and high, 13 females from control and low treatment and 14 females from high treatment group. To quantify the focal fish behaviours, videos were blindly scored using the behavioural software BORIS (version 9.0.8; Friard & Gamba 2016), a software that allows for manual logging of events and durations of different fish movements and behaviours. A behavioural ethogram (Table 1) was used, containing the continuous state events: time to emerge from home shell (because all focal fish start their trials hiding within their shells), time spent in the social zone, asocial zone, and how long time they spent in the home shell, social shell, and asocial shell. Duration of time spent in the middle zone was calculated by subtracting time spent in social and asocial slides from total time. Proportion of total time spent in social, asocial and middle zones was then calculated. The standard length of each fish was measured from still frames in Image J (version 1.54g; Schneider et al. 2012) relative to known distances within each tank.

Table 1. Ethogram used for social scoring.

Behaviour	**Description**
Time to emerge	The time it takes for the focal fish to emerge from its home shell
Social zone	Time the focal fish spends within a 5 cm zone from the glass divider next to the stimulus pair.
Asocial zone	Time the focal fish spends within a 5 cm zone from the glass divider next to the empty compartment.
Home shell	Time the focal fish spent hiding within the home-shell/the middle shell.
Social shell	Time the focal fish spent hiding in the shell within the social zone.
Asocial shell	Time the focal fish spent hiding in the shell within the asocial zone.

2.3 Statistical analysis

The statistics were carried out using R (version 4.4.3; R Core Team 2025) and packages igraph (Csardi & Nepusz 2006), ggraph (Pedersen 2024), ggplot2 (Wickham 2016), dplyr (Wickham et al. 2023), lmtest (Zeileis & Hothorn 2002), glmmTMB (Brooks et al. 2017), performance (Lüdecke et al. 2021), DHARMa (Hartig 2024), tidyverse (Wickham et al. 2019) and emmeans (Lenth 2025).

2.3.1 Modularity

To analyse the spatial arrangement of shells and group spacing of *N. multifasciatus* I analysed the modularity as a metric. First the pairwise distances between all shells were calculated (using the Pythagorean theorem). These distances, initially measured in pixels, were converted to centimetres using measurements taken in ImageJ (version 1.54g), based on the pixel width and known width of the test tank. A proximity matrix was then constructed so that shells closer to each other had stronger connections (by taking the inverse of the distance between each pair of shells). To identify clustering within the shell spatial networks, I applied the Walk Trap Algorithm, a random walk-based community detection method within the Igraph package (Csardi & Nepusz 2006). This algorithm was used separately for each tank and day, and a higher modularity score indicates that shells in the tank are clustered together more closely. A generalized linear mixed effects model (GLMM) was then fit with treatment and day as fixed effects, modularity as the response variable, and a gaussian distribution. Tank number was included as a random intercept. Normality, homogeneity of variance, and model fit were tested using diagnostic plots from the package's performance (Lüdecke et al. 2021), DHARMa (Hartig 2024), and Shapiro-Wilks test (Shapiro & Wilk 1965). A post hoc test was then performed (emmeans) (Lenth 2025) to estimate trend differences of modularity over time between the three treatments.

2.3.2 Sociality

To examine if exposure to diazepam affects sociality, and if it is dose-dependent, the sociality data were individually analysed (Wickham 2016) and GLMMs (Brooks et al. 2017). In all models, treatment and sex were included as fixed effects, and standard length as a continuous covariate. I included tank ID and stimulus pair ID as random intercept effects. This model structure was applied to the following response variables (with the distribution used in parentheses):

1. Total duration of time to emerge from the home shell (gaussian distribution).

2. Proportion of total time spent on the social zone (beta distribution).

3. Proportion of total time spent in the asocial zone (beta distribution).

4. Proportion of total time spent in the middle, (in between each zone, beta distribution).

5. Number of zone changes given by the total number of times the fish went into the social and asocial zone (negative binomial distribution).

For all models, normality, homogeneity of variance, and model fit was tested using the packages performance (Lüdecke et al. 2021), DHARMa (Hartig 2024). A likelihood Ratio Test was performed to check if interaction between treatment and sex significantly improved model fit (drop1) (Appendix 1, Table C). If it did not, then the interaction was removed, and treatment and sex were kept as separate main effects. A post hoc test, emmeans (Lenth 2025) was then performed to estimate the contrast between treatments and sex (if there was an interaction) and between treatments if there was no interaction (Appendix 1, Table C).

For the duration of time to emerge data (response variable 1), I did a Shapiro-Wilks test (Shapiro & Wilk 1965) instead of using performance (Lüdecke et al. 2021). Due to skewness in the data a $\log_e$ transformation was conducted. For the zone change data (response variable 5) three different GLMMs, one with Poisson distribution and two with different types of negative binomial GLMMs (nbinom1 and nbinom2) was tested. The models were compared using Akaike Information Criterion (AIC) to determine which model fit the data best, the GLMM with Poisson distribution (df = 9, AIC = 862.86), nb1 (df = 10, AIC = 665.2693), and nb2 (df = 10, AIC = 663.57), nb2 was chosen and used during modelling.

2.4 Ethics

All work done in this thesis was performed under the approval Dnr: A-5-2023 from Jorbruksverket. To work with live fish, the following courses have been completed: Svensk lagstiftning and etik, djurskydd och 3R as well as Laboratory Animal Science - Fish (both theoretical and practical parts). The required safety paper for working in the lab as well as introduction to the lab have been reed. A thorough review of safety protocols, handling, caretaking, lab methods and work routines has been done. All this to ensure both the animal welfare and safety for myself and others in the lab.

3. Results

3.1 Modularity

A total of n = 105 still images were collected from all 21 treatment tanks at five different time points. On day 6, no significant differences were observed between the control group and low treatment (all $P > 0.54$; Appendix 2, Table D; Figure 2 b). In general, modularity increased over time for all treatments (Est ± SE = 0.0032 ± 0.00040, z = 8.55, $P < 0.0001$), but this increase depended on treatment. Post hoc comparisons showed a significant difference in modularity between the control and high treatment (Est ± SE = 0.0018 ± 0.00053, t = 3.44, $P = 0.0024$; Figure 2 a & b), where modularity increased to a lesser extent than the control group. No significant differences were found between the control and low treatment or the low and high treatment (all $P > 0.85$); Appendix 2, Table D). However, the low treatment was intermediate between the control and high groups (Figure 2 a & b). There was a significant interaction between treatment and time (df = 2, AIC = -591.83, LRT = 11.29, $P = 0.0035$)

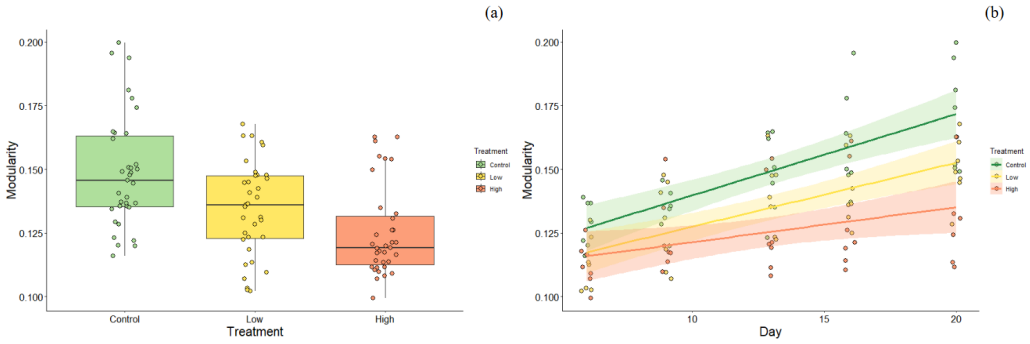

Figure 2. Modularity of shell arrangements in the testing tanks, plotted by treatment control (green), low (yellow), and high (red). a) Boxplots show median and variation of modularity from all five days. Where the horizontal lines showing medians, boxes showing interquartile range and points showing individual observations. b) Lines show average modularity trends over time with 95 % confidence intervals for modularity over time. Points are individual observations

3.2 Sociality

A total of n = 79 fish from 21 tanks, divided across the three treatment groups (13 males from each treatment, 13 females for control and low, and 14 females from high treatment), were included in the sociality trials.

3.2.1 Time to emerge

Of the total 79 fish, six did not emerge from their home shell during the trial time. When excluding the ones that didn't emerge, the mean time to emerge was (mean ± s.d.) 792 ± 511 seconds for the control group, 770 ± 700 seconds for low and 329 ± 334 seconds for high treatment. Sex and standard length alone had no significant effect on emergence time (all $P > 0.30$; Table E). There was an interaction between treatment and sex that significantly improved model fit (LRT $P = 0.049$; Table C). In a post hoc comparison test, we therefore contrasted the treatments within each sex. Females in the high treatment had significantly shorter emergence time than both the control group (Est ± SE = 1.65 ± 0.36, z = 4.64, $P < 0.0001$) and low treatment (Est ± SE = 1.60 ± 0.36, z = 4.47, $P = 0.0001$). For males no significant differences between treatment were found (all $P > 0.11$, Table E).

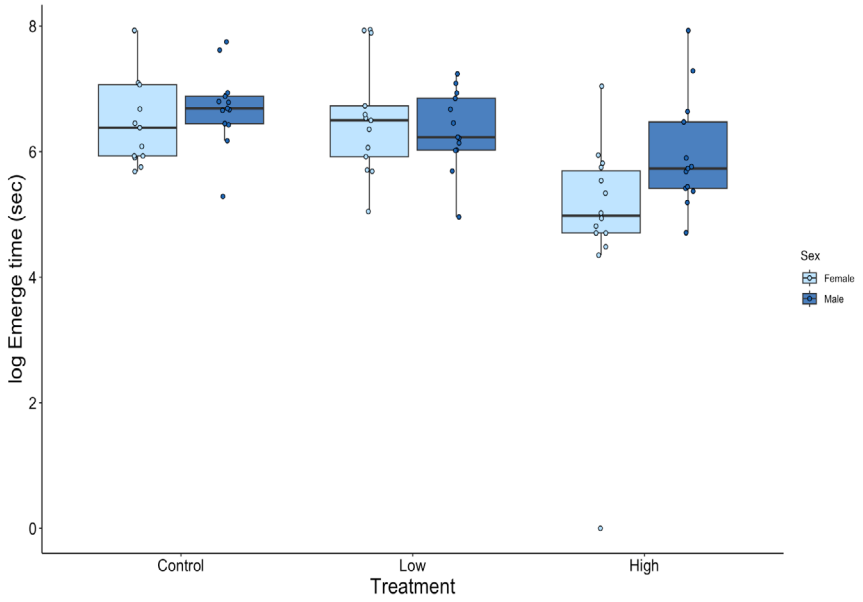

Figure 3. Log-transformed emergence time, plotted by treatment and sex, females (light blue) and males (dark blue). Where the horizontal lines showing medians, boxes showing interquartile range and points showing individual observations.

3.2.2 Proportion of time spent in zones

Of the total 79 fish, 73 entered the social zone. The main effects of GLMM showed that standard length (SL) had no significant effect on proportion spent in the social zone ($P > 0.057$; Table F). The likelihood ratio test showed the interaction between treatment and sex improved model fit ($P = 0.021$; Table C). The post hoc contrasts for treatment within each sex showed that females in the high treatment spent significantly more time in the social zone compared to the control group (Est ± SE = -1.53 ± 0.35, z = -4.43, $P < 0.0001$) and low treatment (Est ± SE = -1.53 ± 0.35, z = -4.43, $P = 0.0025$; Figure 4a). There was no

significant difference between the control group and low treatment, though low spent more time on the social zone than the control group ($P > 0.60$). For males there was no significant difference between treatments (all $P = 0.63$; Table F; Figure 4a).

Of the total 79 fish 70 of them entered the asocial zone. Sex and standard length had no significant effect on time spent in asocial zone ($P > 0.062$; Table F). There were no significant differences between the treatments (all $P > 0.15$; Table F; Figure 4b).

All fish started in the middle zone and therefore spent some of the trial time there. Males spend significantly less time in the middle zone than females (Est ± SE = -0.83 ± 0.26, z = -3.20, $P = 0.0014$) (Figure 4c). The standard length also had a significant effect on time spent in the middle zone (Est ± SE = 0.41 ± 0.21, z = 1.99, $P = 0.047$) meaning that bigger fish spend more time in the middle zone. When comparing the treatments using post hoc contrasts, we found that fish from the control group spent more time in the middle zone compared to fish from the high treatment (Est ± SE = 1.001 ± 0.24, z = 4.18, $P = 0.0001$). We also found that low treatment fish spend significantly more time in the middle zone than high treatment fish (Est ± SE = 0.74 ± 0.23, z = 3.26, $P = 0.0033$). There was no significant difference between the control group and low treatment ($P = 0.51$; Table F; Figure 4c).

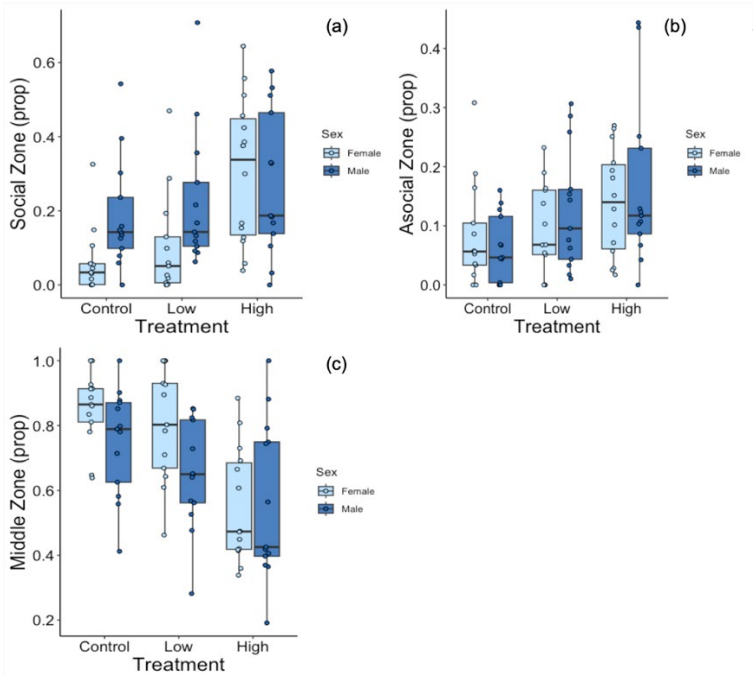

Figure 4. Proportion of total time spent in different zones per treatment and sex, females (light blue) and males (dark blue). Where the horizontal lines showing medians, boxes showing interquartile range and points showing individual observations. a) social zone, b) asocial zone, and c) middle zone.

3.2.3 Zone changes and shell use

Zone changing, defined as the number of times the fish entered the social zone or asocial zone, varied between treatments and sexes, with male fish entered zones more frequently than females (Est ± SE = 0.41 ± 0.13, z = 3.23, *P* = 0.0012) (Figure 5). Standard length did not significantly affect zone changing behaviour (P = 0.68; Appendix 2, Table G). Post hoc comparisons show contrasts among treatments where fish in the control group changed zones significantly less than those in low (Est ± SE = -0.32 ± 0.11, z = -2.89, *P* = 0.011) and high treatment (Est ± SE = -0.37 ± 0.11, z = -3.49, *P* = 0.0014). No significant difference was observed between low and high treatment (*P* = 0.89; Appendix 2, Table G; Figure 5).

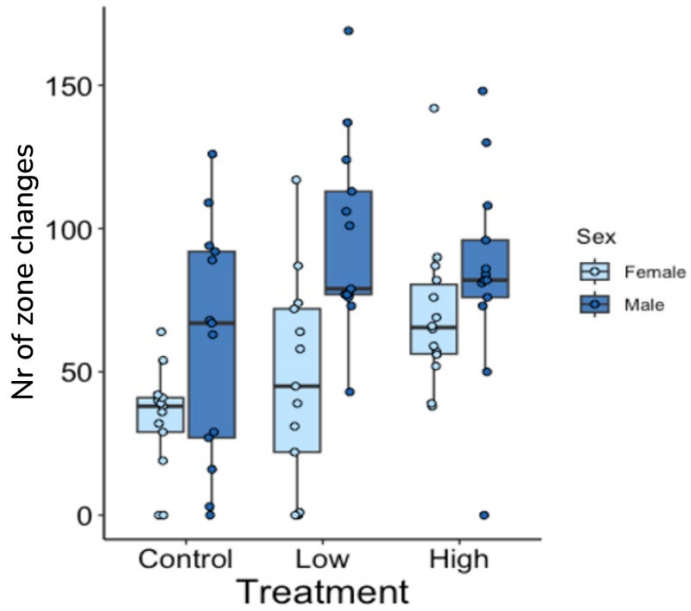

Figure 5. Number of times the fish changed zone per treatment and sex, females (light blue) and males (dark blue). Where the horizontal lines showing medians, boxes showing interquartile range and points showing individual observations.

Looking further into the shell use 73 out of 79 fish entered the home shell at least one time post emergence. For the social shell 45 out of 79 fish entered, and for asocial shell 68 of 79 fish entered. Table 2 summarises how fish used the different shells in the testing tanks across sex and treatment. In general, females entered the shell more frequently than males, while the number of shell entries varied among treatments (Table 2).

Table 2. How many fish entered the shells during the trials and the mean number (M) ± standard deviation (sd) of times the fish entered different shells across treatment and sex.

Treatment	Sex	Home shell entries	Home shell M ± sd	Social shell entries	Social shell M ± sd	Asocial shell entries	Asocial shell M ± sd
Control	Female	11	5 ± 4.08	4	27.9 ± 12.8	11	6.91 ± 5.34
Control	Male	12	7 ± 5.70	5	11.4 ± 8.58	9	5.11 ± 3.59
Low	Female	11	6.25 ± 7.37	4	19.5 ± 16.7	10	8.0 ± 5.08
Low	Male	13	3.1 ± 1.97	10	19.8 ± 14.8	12	7.50 ± 7.50
High	Female	14	14 ± 18.9	13	27.6 ± 12.5	14	11.70 ± 8.65
High	Male	12	6.56 ± 3.50	9	15.4 ± 9.77	12	11.20 ± 5.75

4. Discussion

Pharmaceutical pollution is a growing environmental problem, especially as anxiolytic drugs like diazepam are increasingly found in aquatic environments (Brodin et al. 2014; Wilkinson et al. 2022). In this thesis, I investigated whether diazepam affects social attraction and group spacing in the shell dwelling cichlid *Neolamprologus multifasciatus*. As predicted, the fish exposed to diazepam showed a difference in group spacing with reduced territorial clustering as well as increased social attraction, and there was a dose-dependent effect on some of the parameters.

Shell clustering (modularity) increased over time in all treatments, but the increase was significantly weaker in the high group compared to control. The weaker increase suggests that diazepam led to groups where fish were spaced farther apart from one another. This result may be best explained by increased activity or behavioural disinhibition. Fish under the influence of diazepam may have moved more or used more space, leading to a more dispersed group spacing without necessarily indicating reduced social attraction. In the sociality trials, fish exposed to high doses of diazepam emerged faster and spent more time in the social zone, close to the stimulus fish. Stronger social attraction may be a result of reduced anxiety and increased boldness, making the fish more likely to initiate social contact. The fish also changed zones more often than control fish, indicating more activity. These results are in line with earlier studies that show benzodiazepines can increase boldness and overall activity (Brand et al. 2025; Brodin et al. 2013; McCallum et al. 2021). The data from shell use showed that high treatment fish went into both social and asocial shell more often than control fish. Since shells are important for *N. multifasciatus* for both shelter and reproduction, this change in behaviour could reflect altered perception of risk or an increase in activity and boldness (Bose et al. 2021).

My results also showed that diazepam had a stronger behavioural effect on females than on males. Females in the high treatment emerged faster and spent more time in the social zone compared to control and low treatment females. Males, although generally more social and exploratory overall, did not show clear dose-dependent differences. The increase in social attraction among high treatment females reduced the behavioural gap between the sexes. This could be due to a ceiling effect, where males already exhibit high baseline boldness, limiting further behavioural change. Alternatively, females may be more sensitive to the drug due to sex-specific differences in receptor density, hormone levels, or body size, which could influence absorption (Michelangeli et al. 2022; Wilson et al. 2004). Similar sex differences have been observed in mice, where females

exposed to diazepam spent more time in open areas, suggesting stronger anxiolytic effects compared to males (Wilson et al. 2004)

My findings suggest that diazepam interferes with behaviours important for maintaining close group spacing. One reason animals form tight groups is to reduce predation risk, the "safety in numbers" effect (Ward & Webster 2016). If diazepam reduces fear and anxiety, the perceived need to stay close to others may also decrease, leading to more disperse group spacing even when social attraction remains. Michelangeli et al. (2022) describe how chemical pollutants can affect group dynamics, by altering fear responses and social signalling. This aligns closely with what I observed in my experiment, where diazepam influenced both spatial clustering and social attraction. While social attraction was highest in high treatment, shell clustering showed the weakest increase. At first, this might seem contradictory. However, these outcomes likely reflect different underlying behavioural mechanisms. Diazepam may increase general activity and disinhibition, making fish more likely to approach others in some situations, while also causing them to move more and use a larger area of the environment. This could explain why clustering increased to a lesser extent, even though social attraction increased in a different behavioural context.

Additionally, the sociality trials in this experiment involved unfamiliar fish, whereas the shell clustering data came from groups of familiar individuals. How familiarity with conspecifics influences behavioural responses under diazepam exposure remains unclear, but it may help explain some of the differences observed between the two tasks. It is important to note that the changes observed here primarily occurred in the high treatment, which is above the concentration of diazepam measured in the environment. Still, I have shown that pharmaceuticals like diazepam can interfere with social behaviours, this can give an insight into sublethal effects of pharmaceutical contamination under more extreme or cumulative exposure scenarios. This study give an important view of how long-term exposure of diazepam affects social behaviour under a complex social environment.

A limitation of this thesis is that the behaviour of the focal fish in the sociality trials may have been influenced by the behaviour of the stimulus pair. Although only a limited numbers of stimulus pairs were used within trials, individual differences in activity level, aggression, or attractiveness among stimulus fish could still have affected focal fish responses. However, this variation was accounted for statistically by including stimulus pair ID as a random effect in the generalized linear mixed models. To improve standardization in future studies, one possible solution could be the use of pre-recorded or computer-generated video stimuli to control for variation in stimulus behaviour. However, such an

approach would need further validation to ensure that the focal fish respond to video stimuli. This experiment was conducted in a controlled lab setting, which makes it easier to study specific effects, but also means it does not fully represent how things work in the wild. In nature, fish face changing conditions, predators, and often more than one contaminant at a time (McCallum et al. 2021; Wilkinson et al. 2022). Future research should investigate long term exposure, and multi-contaminant effects, ideally under field conditions.

4.1 Conclusion

This thesis shows that pharmaceuticals such as diazepam can have a disruptive impact on spatial grouping and social attraction in *Neolamprologus multifasciatus*. The observed increase in social attraction and zone change in high treatment fish, paired with a weaker increase in spatial clustering, suggests that diazepam alters activity levels in exposed fish in ways that disrupt the spatial structure of their social groups. These changes in behaviour, although observed under high concentrations of diazepam in a controlled laboratory environment, raise ecological concerns about the potential consequences of pharmaceutical pollutants and how they may affect freshwater ecosystems, though in nature living in groups is vital for avoiding predators and defending resources. Understanding how substances like diazepam alter social groups is crucial when assessing their environmental impact and long term effects.

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Popular science summary

Every day, medicines we humans take, like antianxiety drugs, pass through our bodies and can end up in wastewater. Although wastewater treatment plants remove many pharmaceuticals traces of these often remain, enter rivers and lakes where they can affect animals living there. One such drug is diazepam (commonly known as Valium^{TM}), which is used to treat anxiety in humans. My thesis explores how diazepam influences group spacing and social attraction of a small, highly social fish called *Neolamprologus multifasciatus (N. multifasciatus)*. This fish lives in shells on the bottom of Lake Tanganyika in Africa, where they form structured social groups and clear territories.

To examen the effect of diazepam on *N. multifasciatus* I exposed them to three different concentrations: no diazepam (control), low dose, and a high dose. Their behaviour was observed in two ways. To study group spacing, I looked at the spatial arrangement of shells that the fish do when forming territories. To measure social attraction, each fish was placed in a separate tank where it could choose to spend time near other fish or be alone. I also looked at how fast the fish swam out of its shell at the start, how they used the available space in the tank, and how often they visited the social versus non-social zones.

The results showed that diazepam exposed fish, especially in high doses left their shell shelters more quickly, spent more time near other fish, and moved around more. At the same time, their shell clusters were more spread out, showing lower group spacing. These effects were especially clear in female fish. The results suggest that when human medication end up in the aquatic environment it can affect the way fish behave socially, mating patterns, and even survival in the wild. Even though this study was done in a lab, and the effects were most pronounced at the high dose, it can give important clues about what might happen in the wild if pharmaceuticals continue to enter waterways.

Appendix 1

Table A. The mean measures of the parameters that was controlled during water quality checks and how many times it was measured.

	Control	**Low**	**High**
Temp mean ± sd	26.11 ± 0.68	27.11 ± 0.60	26.91 ± 0.46
Temp Count	14	11	12
O2 mean ± sd	10.001 ± 0.28	9.83 ± 0.33	9.92 ± 0.23
O2 Count	6	5	7
Nitrite mean ± sd	0 ± 0	0 ± 0	0 ± 0
Nitrite Count	18	13	13
Nitrate mean ± sd	15.14 ± 7.044	14.62 ± 5.76	12.50 ± 5.00
Nitrate Count	18	13	13
GH mean ± sd	8.33 ± 1.56	6.96 ± 0.66	6.88 ± 1.0032
GH Count	18	13	13
KH mean ± sd	6.00 ± 0.59	6.15 ± 0.55	5.92 ± 0.28
KH Count	18	13	13
Cl mean ± sd	0 ± 0	0 ± 0	0 ± 0
Cl Count	18	13	13
pH mean ± sd	7.75 ± 0.64	7.99 ± 0.48	8.18 ± 0.39
pH Count	17	11	11

Table B showing stimulus pairs used for sociality trials and their standard length.

Stimulus fish pair	**Test tank (day 43-46)**	**Test tank (day 47-49)**	**Male SL (cm)**	**Female SL (cm)**
1	Tank 1 (control)	Tank 4 (low)	3.6	3.2
2	Tank 1 (control)	-	2.9	2.5
3	Tank 4 (low)	Tank 6 (high)	3.7	2.9
4	Tank 4 (low)	-	2.9	2.4
5	Tank 5 (high)	-	3.4	2.4
6	Tank 6 (high)	-	3.0	2.4
7	-	Tank 1 (control)	3.9	2.6
8	-	Tank 2 (control)	3.1	2.5
9	-	Tank 3 (low)	3.2	2.5
10	-	Tank 5 (high)	3.6	2.3

Table C. Likely hod ratio test for social interaction across models

Interaction (drop1)	**SL (df 1)**	**Treatment: sex (df 2)**
Time to emerge	AIC = 230.76, LRT = 0.95, *P* = 0.33	AIC= 233.86, LRT = 6.28, ***P* = 0.049**
Social zone	AIC =-51.264, LRT =3.58, *P* = 0.058	AIC= -49.14, LRT = 7.71, ***P* = 0.021**
Asocial zone	AIC = -92.88, LRT = 2.71, *P* = 0.26	AIC= -93.24, LRT = 0.35, *P* = 0.55
Middle zone	AIC =-10.009, LRT=3.13, *P* = 0.077	AIC= -11.62, LRT = 3.52, *P* = 0.17
Zone change	AIC = 661.87, LRT = 3.56, *P* = 0.59	AIC= 663.14, LRT = 0.29, *P* = 0.17

Appendix 2

Table D. The output of one GLMM for the modularity data indicating clustering in the shell network, significant P values are marked bold, including emmtrends post hoc contrasts among treatment groups for slope differences over time.

	Estimate ± SE	***z***	***t***	***P***
Effect				
Low	-0.0055 ± 0.0090	-0.61	-	0.55
High	-0.00020 ± 0.0090	-0.020	-	0.98
Day	0.0032 ± 0.00040	8.55	-	**< 0.0001**
Low Day	-0.00070 ± 0.00050	-1.29	-	0.20
High Day	-0.0018 ± 0.00050	-3.44	-	**0.00058**
Post hoc contrasts				
Control - Low	0.00068 ± 0.00053	-	1.29	0.40
Control - High	0.0018 ± 0.00050	-	3.44	**0.0024**
Low - High	0.0011 ± 0.00053	-	2.15	0.85

Table E. Results from GLMM and post hoc test for time to emerge, significant P values are marked bold, including emmeans post hoc contrasts among treatment groups per sex.

	Estimate ± SE	**z**	**P**
Effect			
Low	-0.05 ± 0.37	-0.14	0.89
High	-1.65 ± 0.36	-4.64	**< 0.0001**
Male	-0.10 ± 0.45	-0.21	0.83
SL	0.25 ± 0.24	1.033	0.3014
Low Male	-0.31 ± 0.52	-0.60	0.55
High Male	0.92 ± 0.51	1.80	0.072
Post hoc contrasts			
Female			
Control - Low	0.051 ± 0.37	0.14	0.99
Control - High	1.65 ± 0.36	4.64	**< 0.0001**
Low - High	1.60 ± 0.36	4.47	**0.0001**
Male			
Control - Low	0.36 ± 0.36	0.99	0.59
Control - High	0.74 ± 0.36	2.03	0.11
Low - High	0.38 ± 0.36	1.05	0.55

Table F. Proportion of total time spent significant values are marked with bold text. significant P values are marked bold, including emmeans post hoc contrasts among treatment groups per sex.

	Proportion spent in:	**Estimate ± SE**	**z**	***P***
Effect				
Low	Social zone	0.37 ± 0.38	0.97	0.33
High	Social zone	1.53 ± 0.34	4.43	**< 0.0001**
Male	Social zone	1.57 ± 0.43	3.65	**0.00026**
SL	Social zone	-0.45 ± 0.23	-1.902	0.057
Low Male	Social zone	-0.24 ± 0.50	-0.48	0.63
High Male	Social zone	-1.23 ± 0.47	-2.60	**0.0094**
Post hoc contrasts				
Female				
Control - Low	Social zone	-0.37 ± 0.38	-0.97	0.60
Control - High	Social zone	-1.53 ± 0.36	-4.43	**< 0.0001**
Low - High	Social zone	-1.16 ± 0.35	-3.33	**0.0025**
Male				
Control - Low	Social zone	-0.13 ± 0.32	-0.41	0.91
Control - High	Social zone	-0.30 ± 0.33	-0.92	0.63
Low - High	Social zone	-0.17 ± 0.32	-0.54	0.85
Effect				
Low	Asocial zone	0.31 ± 0.31	1.018	0.31
High	Asocial zone	0.57 ± 0.31	1.85	0.064
Male	Asocial zone	-0.046 ± 0.27	-0.17	0.87
SL	Asocial zone	0.12 ± 0.23	0.50	0.62
Post hoc contrasts				
Control - Low	Asocial zone	-0.31 ± 0.31	-1.018	0.57
Control - High	Asocial zone	-0.57 ± 0.31	-1.85	0.15
Low - High	Asocial zone	-0.26 ± 0.30	-0.87	0.66
Effect				
Low	Middle zone	-0.26 ± 0.24	-1.10	0.27
High	Middle zone	-1.0014 ± 0.24	-4.18	**< 0.0001**
Male	Middle zone	-0.83 ± 0.26	-3.20	**0.0014**
SL	Middle zone	0.413 ± 0.21	1.99	**0.047**
Post hoc contrasts				
Control - Low	Middle zone	0.26 ± 0.24	1.10	0.51
Control - High	Middle zone	1.001 ± 0.24	4.18	**0.0001**
Low - High	Middle zone	0.72 ± 0.23	3.26	**0.0033**

Table G. Number of times the fish entered social and asocial zone (sum); significant P values are marked bold.

	Estimate ± SE	**z**	***P***
Effect			
Low	0.32 ± 0.11	2.84	**0.0045**
High	0.37 ± 0.11	3.46	**0.00054**
Male	0.41 ± 0.13	3.23	**0.0012**
SL	0.046 ± 0.10	0.41	0.68
Post hoc contrasts			
Control - Low	-0.32 ± 0.11	-2.89	**0.011**
Control - High	-0.37 ± 0.11	-3.49	**0.0014**
Low - High	-0.048 ± 0.10	-0.47	0.89

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