The impact of the pharmaceutical oxazepam on reproductive endpoints on the cichlid fish *Neolamprologus multifasciatus*

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Abstract

Pharmaceutical waste products are increasingly being detected in the environment where their behaviour-altering effects may impact wild animals. Yet, we have a poor understanding of how these pollutants affect the reproductive success of exposed animals and ultimately the evolutionary selection pressures they experience. In this laboratory experiment, we exposed groups of a highly social fish, *Neolamprologus multifasciatus,* to environmentally realistic concentrations of the pharmaceutical oxazepam over a one-month period. We monitored how offspring production and division of reproduction were affected. This represents one of the first experiments to assess these endpoints under controlled yet naturalistic conditions, where fish were able to create social groups under different exposure scenarios. High doses of oxazepam significantly reduced aggressive behaviours in both reproductive and non-reproductive fish. Larger males and females had higher reproductive success. Specifically high treatment allowed smaller males to become reproductively successful, whereas they were unable to reproduce under control conditions. Reproductive males exhibit more aggressive behaviours than non-reproductive males, while reproductive females show more submissive behaviours than non-reproductive females. Males have larger home ranges, and reproductive females have smaller home ranges than their non-reproductive ones. Overall spatial dynamics, such as home range size and shell selection, remained unchanged by the treatment. These findings suggest that environmentally realistic concentrations of oxazepam can significantly alter social behaviours and reproductive dynamics in *Neolamprologus multifasciatus*.

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Abbreviations

SLU	Swedish University of Agricultural Sciences
RSFs	Resource selection functions

1. Introduction

Pharmaceuticals, encompassing both synthetic and biologically derived substances, exert physiological impacts by stimulating, depressing, or replacing biological functions (Bean et al., 2022). Pharmaceuticals can find their way into the environment through various channels (Boxall et al., 2012), including discharges into surface waters from wastewater treatment systems, aquaculture facilities, and runoff from agricultural fields (Kinney et al., 2006; Ternes et al., 2004; Boxall et al., 2012). Even after ingestion of a pharmaceutical, not all of the compound is metabolized, and a portion of the parent compound will be excreted (Boxall et al.,2012). While pharmaceuticals may be present in the environment at relatively low concentrations, some can persist for long periods of time, and many have been designed to have behavioural effects even at low doses (Arnold et. al., 2014).

Pharmaceuticals' primary use, in humans or animals, is to treat or prevent some disease or a symptom (Boxall et al.,2012). Some substances specifically are designed to modulate humans' behavior, such as antidepressants or anxiolytics (Vaudin et al., 2022). Numerous pharmaceutical drugs, now recognized as environmental pollutants, achieve their therapeutic effects by interacting with the endocrine system and, in many cases, with neural cells (Vaudin et al., 2022). In recent years there has been heightened concern within the scientific community regarding the occurrence, fate, and impacts of pharmaceuticals in aquatic ecosystems. Researchers have shown evidence that the pharmaceuticals that end up in the environment are capable to affect wildlife's behavior (Brodin et al., 2014; Jones et al., 2001). Yet, despite this, the full extent of the consequences of exposure to pharmaceuticals for wildlife remains unclear (Huerta et. al., 2012).

The impacts of pharmaceutical pollutants on behaviour are important to understand, since many behaviours are closely tied to individual fitness and population persistence (Smith & Blumstein, 2008). Behaviour is a cornerstone in shaping the overall fitness of animals, influencing their ability to secure resources, avoid predators, and successfully reproduce (Brodin et. al., 2014). Social behaviours, including communication, cooperation, and competition, have a significant role in establishing and maintaining social structures within populations, enhancing the prospects of survival and reproduction (Werner & Peacor, 2003). A study on juvenile brown trout exposed to the pharmaceutical oxazepam, revealed distinct effects on dominant and subordinate fish (McCallum et al., 2021). The impact of exposure differed between dominant and subordinate fish, resulting in decreased aggression among fish exposed to high doses, and heightened competitive success among subordinate fish exposed to low doses (McCallum et al., 2021). This highlights the complex interplay of environmental contaminants, such as pharmaceutical pollutants, within social structures, underscoring the importance of considering these dynamics in assessing the impact of pollutants on wildlife. Though social behaviours play a crucial role in animal fitness, the impact of pharmaceuticals on these intricate behavioural patterns is not well-explored (Bean et al., 2022). To better understand how pharmaceutical pollution can affect animal fitness, it is essential to assess reproductive outcomes along with the characteristics of individuals who either succeed or fail in reproduction under different exposure levels. Yet, this has rarely been examined in the behavioural ecotoxicological literature to date.

The objective of this thesis was to investigate the effects of the pharmaceutical compound oxazepam on reproductive patterns among groups of *Neolamprologus multifasciatus* (Figure 1.). Oxazepam, classified as a benzodiazepine anxiolytic, is widely prescribed for the management of conditions such as anxiety, sleep disorders, and muscle spasms (Farach et al. ,2012). *N. multifasciatus* ("multis" hereafter) is a group-living social cichlid fish endemic to Lake Tanganyika, in East Africa (Lein & Jordan, 2021). These fish live in mixed-sex restricted-entry social groups with a strong dominance hierarchy, and they exhibit a wide range of social behaviours (Kohler, 1998). Groups consist of a large, aggressive dominant male as well as smaller adult males and females in addition to juvenile individuals. In group dynamics, dominant males monopolize reproduction, securing the majority of mating, while subordinate males receive minimal chances for reproduction. Conversely, females within the group generally have equal reproductive access, albeit primarily mating with the dominant male (Bose et al., 2022a). Multis are a valuable study species because they i) engage in frequent social behaviour, offering numerous opportunities for behaviour-modifying drugs to influence social interactions and potentially therefore also individual fitness, ii) reproduce within tightly-knit social groups, in which group members vary in their reproductive success, allowing comparisons of reproductive success between individuals under different drug exposures, and iii) quickly form social groups and reproduce under laboratory conditions, enabling feasible exposure periods to track behavioural and reproductive consequences of pharmaceutical treatments (Schradin & Lamprecht, 2000).

In this thesis, I analyse behavioural recordings, and data from a laboratory exposure experiment in which *N. multifasciatus* adults were exposed to three different concentrations of oxazepam, and then allowed to form groups, interact, and reproduce with one another over a ~one-month long exposure period. In particular, I investigate how oxazepam exposure affects individual reproductive endpoints, and whether exposure affects the behavioural phenotypes of reproductively successful and unsuccessful individuals in distinct ways. As observed earlier with brown trout, when oxazepam is added to groups with dominant and subordinate members, the usual differences between them become less pronounced (McCallum et al., 2021). This suggests that exposure to oxazepam might affect how social groups in multis typically assign reproduction roles, since the bigger males are usually being favoured (Bose et al., 2022). Therefore, I expect the males exposed to oxazepam to share reproduction more evenly among members show less evidence of reproduction being monopolized by a single big fish (as would be expected in the control groups). Accordingly, I also predict that overall offspring numbers will be higher for exposed fish, as the environment becomes less competitive. In previous research has been suggested the successfully reproductive multis are more aggressive than the non-reproductive ones, especially the males (Suriyampola & Eason, 2015). The production of offspring can lead to intrasexual conflict among adults, as fry compete for space and resources within the territory, intensifying competition during periods of parental care (Kohler, 1998). Hence, I expect that among all the groups, fish that successfully reproduce are likely to show more signs of protecting their territory and being aggressive compared to those that don't succeed. I also expect that oxazepam will reduce the aggression across the treatments. Additionally, it is important to note the possibility of non-monotonic dose responses. In pharmaceutical exposures, it is a common trend for lower doses to moderate levels that have more pronounced effects than higher doses (Beausoleil et. al., 2016).

Figure 1.Image of the shell-dwelling ciclid, Neolamprologus multifasciatus in the lab, taken by Theodora Georgaka (Umeå, 2024).

2. Methods

2.1 Experimental setup

The laboratory conditions were set to mimic a natural day-night cycle with 12 hours of light and 12 hours of darkness. The water temperature in the tanks was maintained at 26 degrees Celsius to provide a stable environment for the fish. A total of 405 adult fish (135 males, 270 females) were uniquely tagged with injectable elastomer to distinguish them from each other. The exposure treatments consisted of three doses: a control dose (0 µg/L oxazepam, N = 9 tanks), a low dose (2 µg/L oxazepam, N = 9 tanks), and a high dose (50 µg/L oxazepam, N = 9 tanks). The low and high doses were chosen to represent concentrations that are ecologically realistic and similar to those found in human therapeutic treatments, respectively. The experiment was conducted in four consecutive rounds to achieve the final sample size. Fifteen fish were put in tanks lined with aquarium sand. Each tank contained 10 females and 5 males (their typical sex ratio in the wild, Kohler,1998; Bose et al. 2022). Oxazepam exposures were prepared by dissolving oxazepam (CAS ID: 604-75-1) in aquarium water to create a stock solution, and the necessary dilution was then added to each tank and stirred with a dip net. Control tanks were also manipulated the same way, but no oxazepam stock solution was added. After initial exposure, three days were given to the groups to acclimate and absorb the pharmaceutical into their tissues and reach a steady-state equilibrium. After these three days, each aquarium was given 30 shells of *N. tanganyiense*, in an evenly spaced grid pattern on the sand, to stimulate group formation, territorial behaviour, and reproduction. The tanks were then exposed to the treatments for a period of 32-36 days. At the end of the experiment, all fish were euthanized with an ice bath followed by cerebral concussion, and then stored frozen for later analyses.

This work was performed with the approval of the Tierschutzgesetzes (TierSchG) Baden-Württemberg, under the ethical permit no. G 19/79.

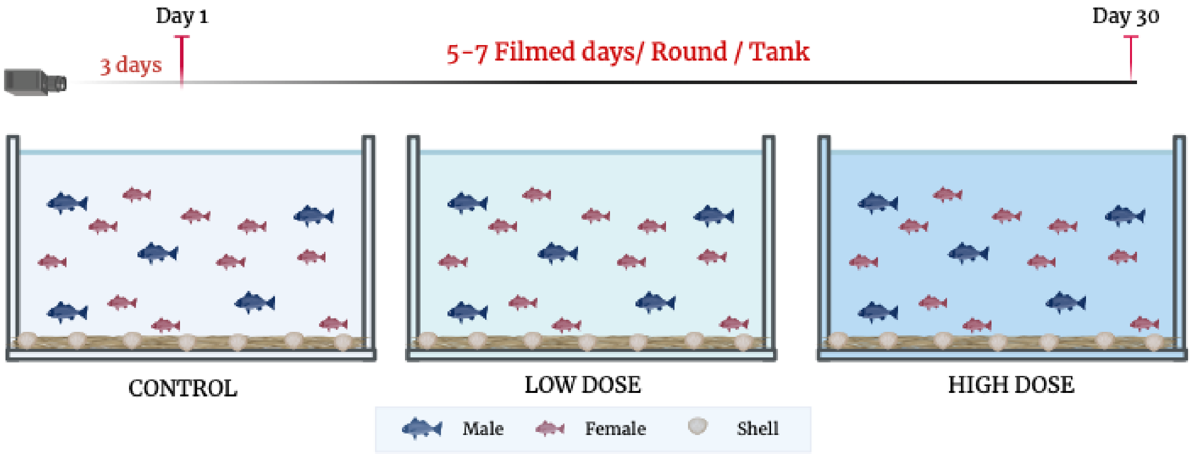

Figure 2. Illustration of the laboratory set-up.Three different treatments, control (0 µg/L), low (2 µg/L) and high (50 µg/L) doses. Each tank contained 15 cichlids (Neolamprologus multifasciatus), five males (blue) and ten females (red). There were also 30 shells of Neothauma tanganyicense snails. The timeline was 3 days of acclimation followed by at least 30 days. The tanks were filmed periodically across the full duration of the experiment.

2.1.1 Body Size and Weight Measurements

At the end of the experiment, body size measurements were collected for both males (N = 135 males, mean ± SD = 35.3 ± 6.1 mm in standard length (SL)) and females (N = 270 females, mean ± SD = 28.3 ± 2.6 mm in SL). Pre-treatment body weight measurements were recorded for males (N = 135, mean ± SD = 1.3 ± 0.7 mm in SL) and females (N = 270, mean ± SD = 0.6 ± 0.2 mm in SL), as well as post-treatment measurements. Males were significantly larger than females (LMM female-male, estimate ± SE = 0.211 ± 0.013, z = 16.21, P < 0.0001, log-transformed), and no differences in body size were observed between different treatment doses (LMM control-low, estimate ± SE = 0.011 ± 0.015, z = 0.75, P = 0.455; control-high, estimate ± SE = -0.006 ±0.016, z = -0.36, P = 0.716; low-high, estimate ± SE = -0.017 ± 0.016, z = -1.08, P = 0.281; log-transformed). Similar results were obtained for body weight, with males being significantly heavier than females (LMM female-male, estimate ± SE = -0.042 ± 0.018, z = -2.32, P = 0.02; control-low, estimate ± SE = 0.04 ± 0.023, z =1.71, P = 0.087; control-high, estimate ± SE = 0.015 ± 0.023, z = 0.67, P = 0.504; low-high, estimate ± SE = -0.025 ± 0.022, z = -1.12, P = 0.261; log-transformed).

2.1.2 Measuring parentage and reproductive success

At the end of the experiment, we took fin clips from all surviving adults as well as any offspring that were produced. Offspring were euthanized. Fin clips and offspring were stored in 99% ethanol and sent for microsatellite genotyping at the University of Graz, Austria. The microsatellite genotyping was performed by following the protocol outlined in Bose et. al., 2022. I quantified reproduction in two ways. I recorded which individuals were reproductively successful based on whether they produced or sired any offspring. And I also recorded the number of offspring produced or sired by each individual as another measure of reproductive success.

2.2 Data collection

2.2.1 Video tracking, Behavioural scoring, Estimation of Territory Size and Assessment of Resource Utilization

On certain days across the exposure period, the fish were video recorded in their tanks by downward-facing cameras (GoPro Hero 8 Black cameras, 2.7K resolution, 30 fps). Each day, one hour of video was recorded for each tank. To manage file sizes, the cameras automatically divided the video recordings into segments lasting 11 minutes and 47 seconds each. Only the 3rd such video segment of each day was used for fish tracking and behavioural analysis.

To track the fish on each video we used an object detection neural network model. Details on the tracking methods can be found in Francisco et al., 2020; Bose et al. 2021 and Rodriguez-Santiago et al. 2020. The tracking process provided X and Y coordinates for each fish in every video frame, creating unique trajectories for each individual across the duration of the video. I manually corrected errors in the track IDs that were generated by the object detection model whenever the fish overlapped with one another in the video frames or temporarily left the field of view. Any gaps in time, such as when fish hide or go temporarily undetected by the model, were filled in using linear interpolation.

Using the individual trajectories, we calculated the total distance moved (in cm) for each fish in the video footage using the 'adehabitatHR' package (version 0.4.21, Calenge & Fortmann-Roe, 2023). We then used this movement data to quantify "territory area" by computing 95% and 50% utilization distributions.

We assessed "resource adherence" for each fish, representing the extent to which fish remained in proximity to the shell resources within their tanks. To achieve this, we calculated resource selection functions (RSFs) with the ‘ctmm’ package in R. This function uses autocorrelation-informed likelihood weighting to reduce autocorrelation associated with high-resolution tracking data, such as ours (Alston, 2023). The resource distribution matched to a binary variable showing whether (1) or (0) shells were present in the fish's immediate surroundings. We used QGIS software (version 3.34.2) to delineate outlines of each shell within every video, thereby capturing the spatial distribution of resources across each aquarium. The RSFs then calculated the correlation between each fish's movement patterns and the distribution of shell resources within each tank.

Finally, we quantified the levels of aggression displayed by each fish based on the individual trajectory tracks. To do this, we used a second neural network model, a behavioural classifier, to identify specific behaviours from our videos, based on kinematic signatures in the fish tracks that it had been trained to recognize as specific behaviours. The development of this neural network was not part of my thesis, but technical details on its methods can be found in Appendix 1. The behaviours that we quantified (in terms of behavioural counts and durations) can be found in Table 1.

Table 1. Ethogram used to score behaviours of Neolamprologus multifasciatus individual (Bose et. al., 2023).

Behaviour	Description
Aggression	
Frontal display	Focal fish faces opponent and extends its opercula and pectoral fins. Often associated with forward and backwards movements of the body
Lateral display	Focal fish positions its body laterally with another fish and adopts a rigid posture. Often accompanied by high amplitude undulation of caudal fin towards the opponent
Bite	Focal fish swims quickly towards opponent, making contact
Chase	Focal fish swims quickly towards opponent, without making contact
Submission	
Submissive display	Focal fish positions its body laterally and may show its belly to opponent. May be accompanied by low amplitude, high-frequency quivering of whole body

2.3 Statistical analyses

I conducted analyses using R (version 4.0.2) and assessed model fits through diagnostics provided by the 'DHARMa' package (Hartig & Hartig, 2017). In all models, interaction terms between predictor variables were initially tested using likelihood ratio tests and included in the final models only if they significantly improved model fits. For all the statistical models I used the 'glmmTMB' package (Brooks et al., 2023).

For the first part of my analysis, I performed the hurdle model approach (Mullahy, 1986) which is divided into two parts. In the first step, a binary choice model is used to calculate the probability when the dependent variable is greater than zero, whereas in the second part, a truncated count data model is used just for observations other than zero (Zeileis et. al., 2008). Initially, I examined how oxazepam treatment (3 level categorical variable: control, low, high) affected reproductive success (binary variable: yes / no for whether the fish produced offspring) separately for males and females using a generalized linear mixed-effects model (GLMM). I also included oxazepam treatment, scaled standard length (mm) and the number of days each individual was alive within a 30-day period as predictors and a random effect of tank's ID nested within round was included to handle data non-independence. The second step of the hurdle approach model was to test how oxazepam treatment affected the amount of the fry produced or sired (count variable) with a GLMM. Again, the oxazepam treatment and the scaled standard length (mm) was used as predictor and a random effect of tank ID nested within round was included.

Further, I conducted three linear mixed models (LMMs) to analyse the duration of behaviours for each individual within the 30-day period. To do that I summed the bite and chase counts as "Attack", the lateral and frontal display as "Display", and the submissive display or "Quiver" stayed as it was. These analyses examined how oxazepam treatment and reproductive success (binary variable: yes / no for whether the fish produced offspring) can affect the duration (continuous variable in sec, log transformed) of each behaviour group. I included oxazepam treatment, reproduction success (binary variable: yes / no for whether the fish produced offspring) , sex (2 level categorical variable: males, females) of the individual that acted the behaviour, and the day (count variable) of the 30-day period as predictors and round, tank's ID, and fish ID as random effects.

Subsequently, I tested the impact of oxazepam treatments and the reproductive success (continuous variable in sec, log transformed) on the territory size (continues variable in cm^2 ,95% utilization distributions in cm^2 ,log transformed) for the last day of the 30-day period using an LMM. I also incorporated scaled standard length, sex as predictors and tank's ID nested within round as random effect.

For the resource selection function analysis, I tested how reproductive success (continuous variable in sec, log transformed) and oxazepam treatment affect the estimate (continuous variable, log-transformed, positive values shows the selection of a shell by the fish and the negative values the opposite) for each fish, at the last day of the experiment, using an GLMM. I included oxazepam treatment, reproduction success and sex as predictors and a random effect of tank's ID nested within Round.

3. Results

3.1 Reproductive success and number of offspring

Table 2. Percentages of individuals that successfully reproduced, in each oxazepam treatment

Sex	Treatment	Reproductive successful individuals (%)	S.E.	Total amount of individuals
Female	Control	25.6	0.0462	90
Female	Low	25.6	0.0462	90
Female	High	28.9	0.0480	90
Male	Control	26.7	0.0667	45
Male	Low	26.7	0.0667	45
Male	High	40.0	0.0739	45

Males

From my analysis, I discovered a significant positive relationship between standardized length and the probability of having produced, where the biggest males had higher probability of having produced than the smaller ones (GLMM, est. ± SE = 1.40 ± 0.31, $z = 4.56$, $p < 0.00001$). Males from the high treatment significantly had increased probability of being reproductively successful compared to the control (GLMM, est. ± SE = 1.24 ± 0.59, $z = 2.11$, $p = 0.035$), whereas low treatment did not show a significant difference from the control (est. ± SE = 0.36 ± 0.57, $z = 0.63$, $p = 0.527$), (Figure 3a).

I observed a significant positive effect of standardized body size on the number of fry produced per sire (GLMM, est. ± SE = 0.495 ± 0.168, $z = 2.95$, $p = 0.00315$). However, the influence of oxazepam treatment on the number of offspring that were produced was not significant. Neither the low (GLMM, est. ± SE = -0.087 ± 0.372, $z = -0.234$, $p = 0.8146$) nor high treatment (GLMM, est. ± SE = 0.214 ± 0.335, $z = 0.639$, $p = 0.5228$) (Figure 3b).

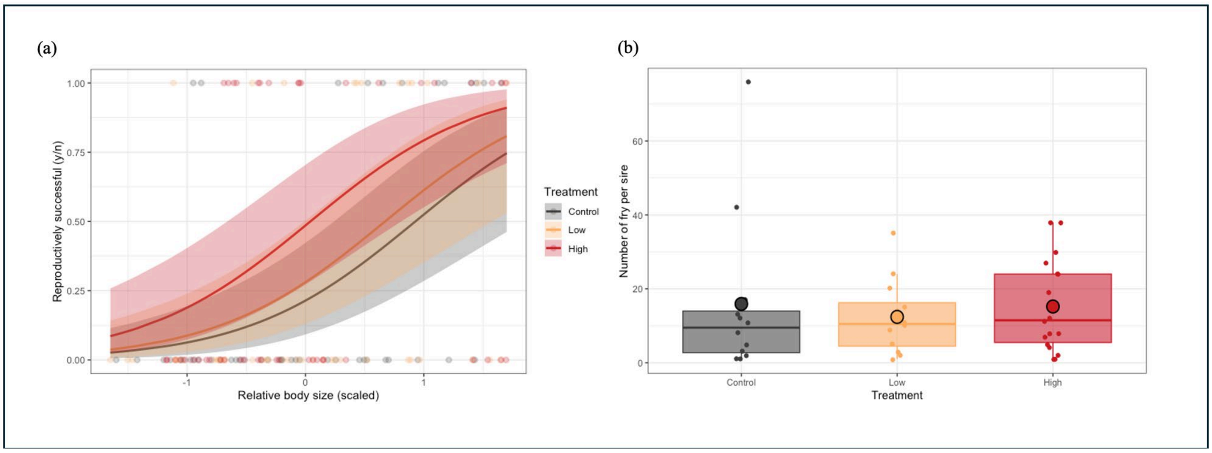

Figure 3.(a) Relationship between relative body size and the probability of having produced across three oxazepam treatments in males. The lines represent model predicted means for each treatment group: Control (black), Low (orange), and High (red). The shaded areas around each line represent the confidence intervals for the respective treatment groups. Individual raw data points, color-coded according to the treatment groups (Control, Low, High). (b) Number of Fry per Sire by Treatment.Each boxplot shows the distribution of the number of fry per sire within each treatment group. The boxes represent the interquartile range (IQR), with the horizontal line inside each box indicating the median number of fry. Whiskers extend to the smallest and largest values. Individual data points are plotted as dots. The larger black lined circles represent the mean number of fry per sire for each treatment group.

Females

The findings from my analysis indicated that larger body size in females significantly increased the probability of having produced (GLMM, est. ± SE = 0.50 ± 0.16, z = 3.09, p = 0.002). While low (GLMM, est. ± SE= 0.038 ± 0.42, z = 0.092, p= 0.926) and high (GLMM, est. ± SE = 0.51 ± 0.45, z = 1.14, p = 0.257) treatments are not significantly different from the control, indicating that treatment does not have a statistically significant effect on reproduction in females. (Figure 4a).

Furthermore, I examined the effects of oxazepam treatment and standardized body size on the number of offspring produced per dam. The results revealed that neither the low treatment (GLMM, est. ± SE = -0.157 ± 0.322, z = -0.487, p = 0.626) nor the high treatment (GLMM, est. ± SE = 0.321 ± 0.286, z = 1.122, p = 0.262) significantly influenced the number of offspring that were produced. Additionally, standardized body size also did not significantly affect the number of offspring that were produced (GLMM, est. ± SE = 0.023 ± 0.099, z = 0.229, p = 0.819), (Figure 4b).

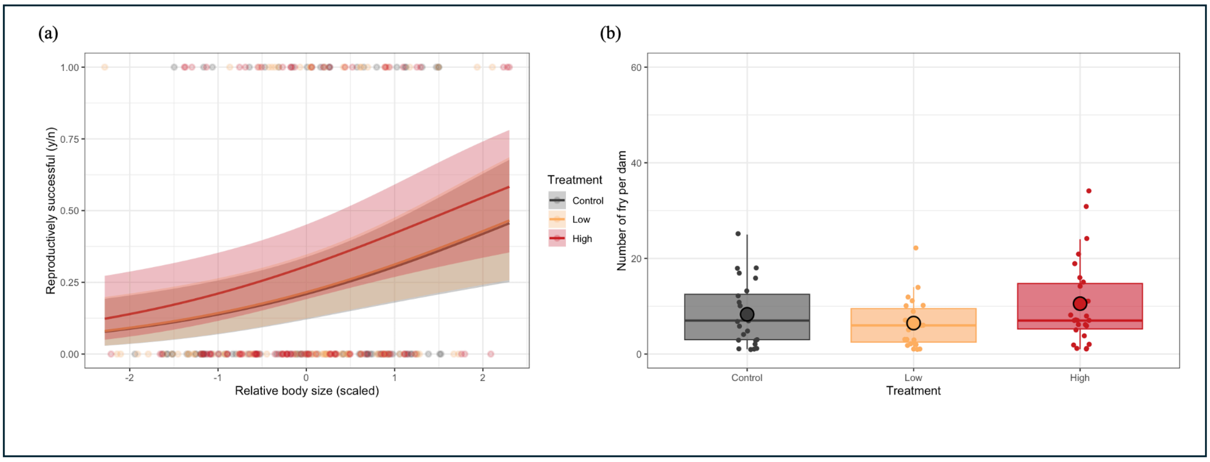

Figure 4.(a) Relationship between relative body size and the probability of having produced across three oxazepam treatments in females. The lines represent model predicted means for each treatment group: Control (black), Low (orange), and High (red). The shaded areas around each line represent the confidence intervals for the respective treatment groups. Individual raw data points, color-coded according to the treatment groups (Control, Low, High). (b) Number of Fry per Dam .Each boxplot shows the distribution of the number of fry per dam within each treatment group. The boxes represent the interquartile range (IQR), with the horizontal line inside each box indicating the median number of fry. Whiskers extend to the smallest and largest values. Individual data points are plotted as dots. The larger black lined circles represent the mean number of fry per sire for each treatment group.

3.2 Reproductive and non-reproductive individuals' behavior

Attack

The analysis showed neither high nor low treatment had any significant relationship with the control treatment. The duration of the "attack" behaviour was significantly higher in males (GLMM, est. ± SE = 0.424 ± 0.065, z = 6.507, $p < 0.0001$) than females. The reproductive individuals had significantly higher duration (GLMM, est. ± SE = 0. 191 ±071, z = 2.696, p = 0.007) than the non-reproductive ones. Also, the interaction between reproduction and sex gave that reproductive males had significantly higher behaviours duration (GLMM, est. ± SE = 0.502 ± 0.099, z = 5.066, $p < 0.00001$) than the reproductive females.

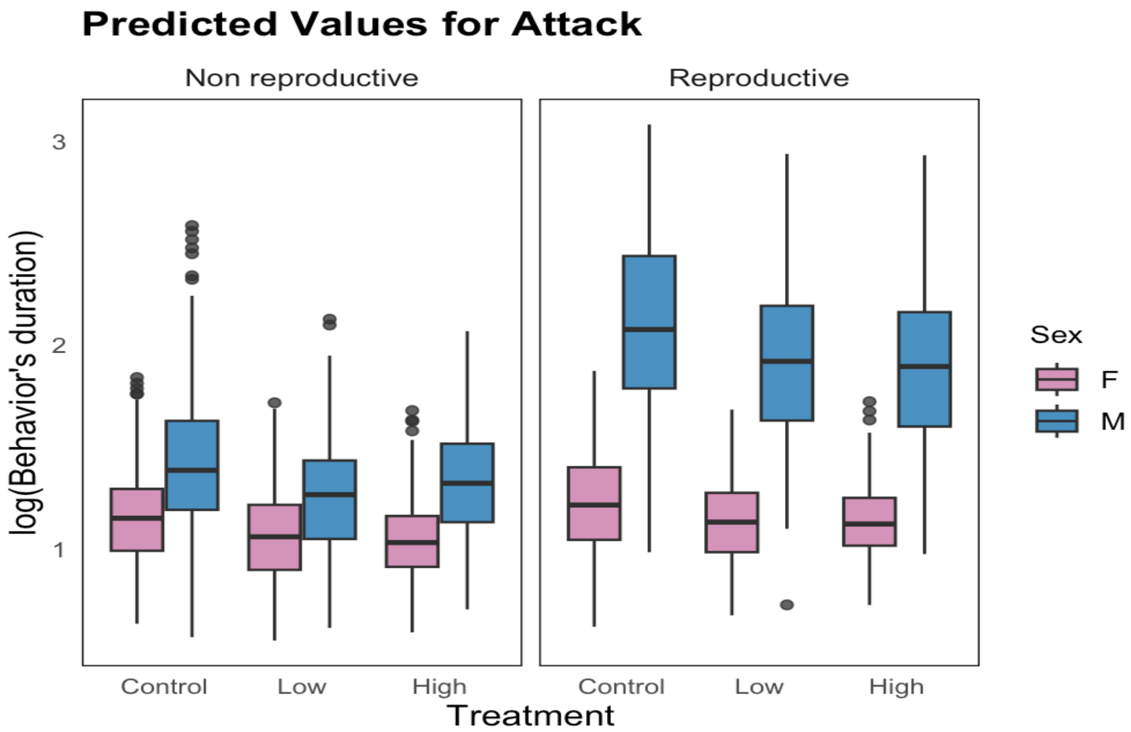

Figure 5. Predicted Values for Attack. The y-axis represents the log-transformed duration of the behaviour. The x-axis represents different treatment levels (Control, Low, and High). Left panel: Non reproductive individuals. Right panel: Reproductive individuals. Males (blue) and female (pink) subjects.The whiskers extend to the most extreme data points not considered outliers, and the points beyond the whiskers are outliers.

Display

The high treatment notably decreased the display behaviour duration (GLMM, est. ± SE = -0.173 ± 0.080, z = -2.17, p = 0.030) when compared to control treatment. But the low treatment had not a significant relationship with control treatment. Furthermore, reproductive males showed increased display behaviour duration (GLMM, est. ± SE = 0.327 ± 0.103, z = 3.17, p = 0.0016) compared to reproductive females.

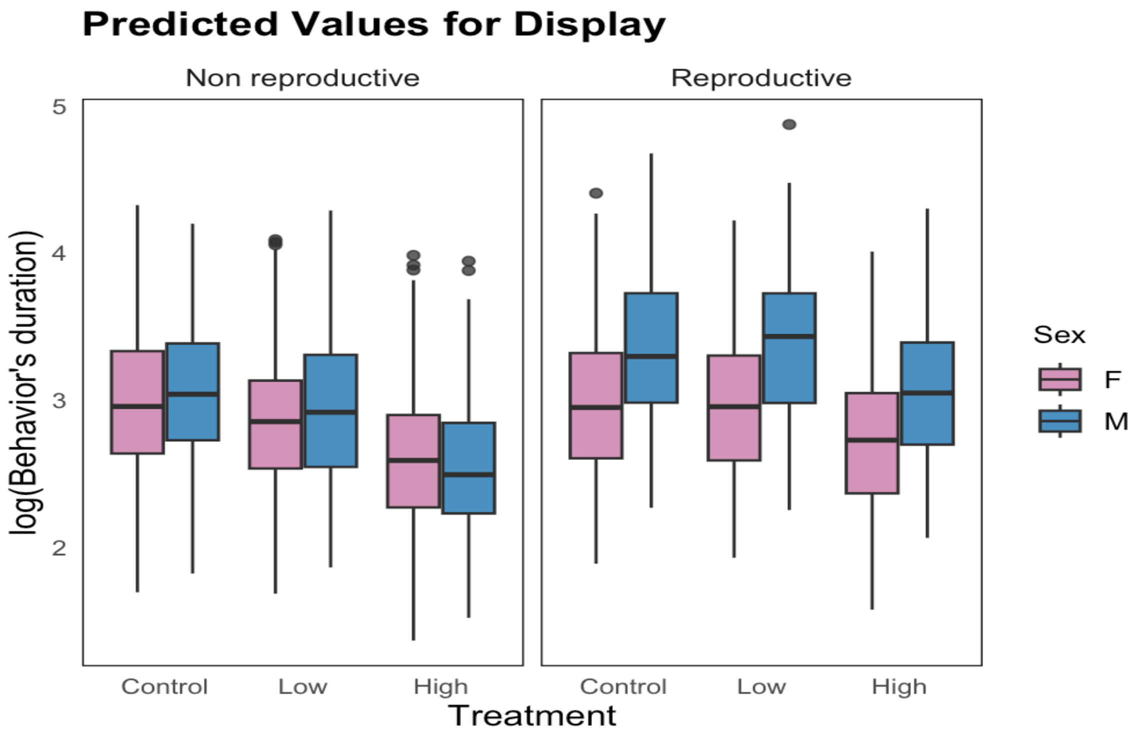

Figure 6. Predicted Values for Display. The y-axis represents the log-transformed duration of the behaviour. The x-axis represents different treatment levels (Control, Low, and High). Left panel: Non reproductive individuals. Right panel: Reproductive individuals. Males (blue) and female (pink) subjects.The whiskers extend to the most extreme data points not considered outliers, and the points beyond the whiskers are outliers.

Quiver

Neither high nor low treatment showed any significance when compared to control treatment. Furthermore, males showed less quiver (GLMM, est. ± SE = -0.527 ± 0.074, z = -7.121, p <0.0001) when compared to females. Reproductively successful female individuals exhibited a significantly longer duration of behaviour (GLMM, est. ± SE = 0.358 ± 0.078, z = 4.622, p <0.0001) then the non-reproductively successful females.

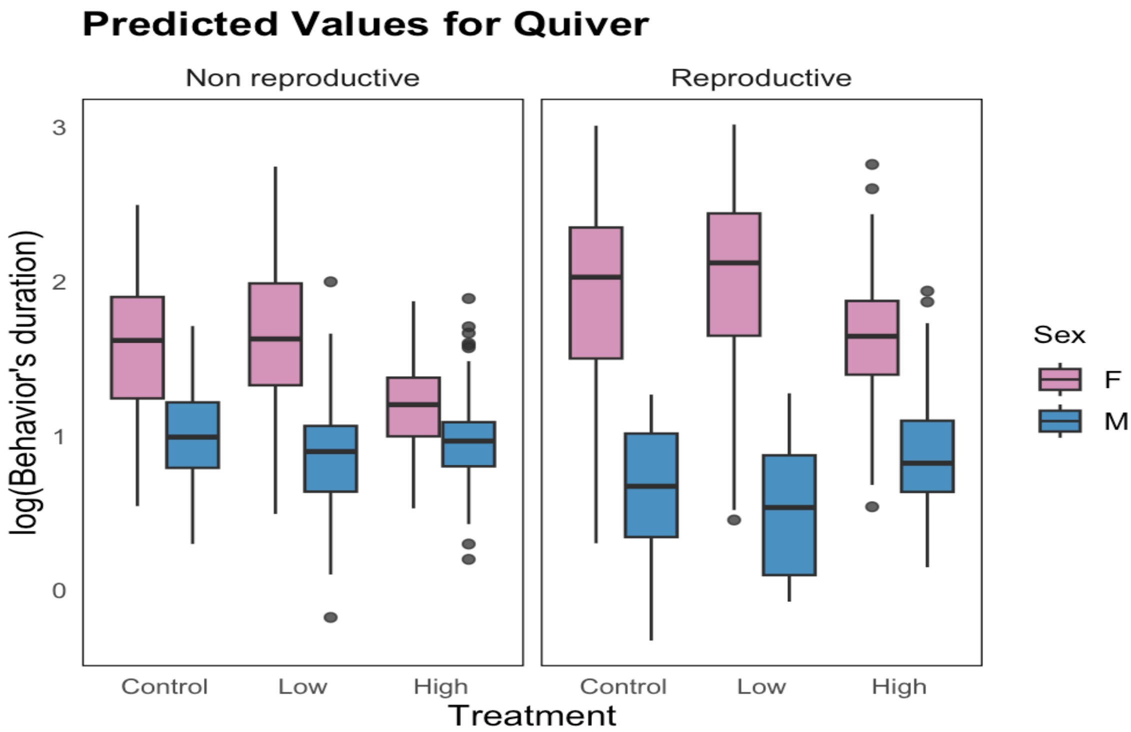

Figure 7.Predicted Values for Quiver. The y-axis represents the log-transformed duration of the behaviour. The x-axis represents different treatment levels (Control, Low, and High). Left panel: Non reproductive individuals. Right panel: Reproductive individuals. Males (blue) and female (pink) subjects. The whiskers extend to the most extreme data points not considered outliers, and the points beyond the whiskers are outliers.

3.3 The home range and the selection of a shell among reproductive and non-reproductive individuals

Home ranges

Males had significantly larger home ranges compared to females (GLMM, est. ± SE = 0.532 ± 0.115, z = 4.62, $p < 0.0001$). Additionally, reproductive females had significantly smaller home ranges compared to non-reproductive females (GLMM, est. ± SE = -0.472 ± 0.107, z = -4.40, $p < 0.0001$). The interaction between sex and reproductive status was highly significant, indicating that reproductive males had notably larger home ranges compared to reproductive females (GLMM, est. ± SE = 1.084 ± 0.190, z = 5.70, $p < 0.0001$).
The body size did not show a significant effect on the home range area (GLMM, est. ± SE = 0.004 ± 0.011, z = 0.33, p = 0.745). Similarly, the treatment conditions, both low (GLMM, est. ± SE = -0.078 ± 0.143, z = -0.55, p = 0.585) and high

(GLMM, est. ± SE = -0.168 ± 0.143, z = -1.17, p = 0.242), didn't show any significance.

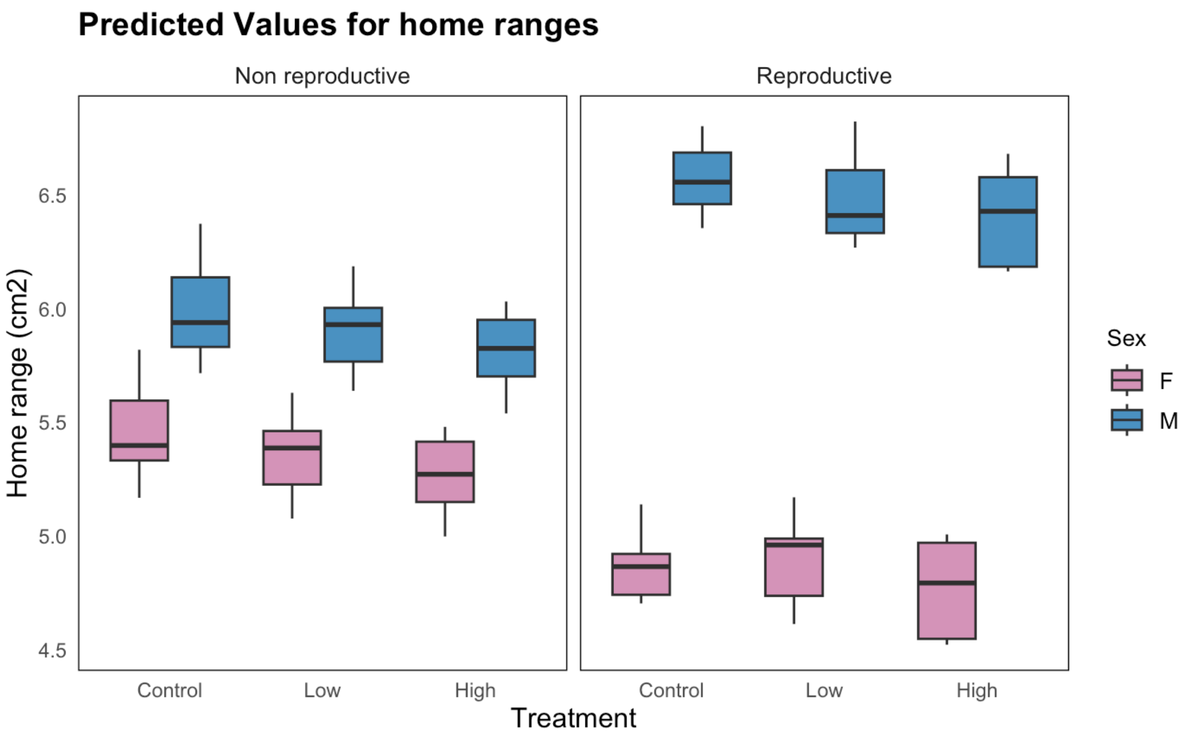

Figure 8. Predicted Values for home ranges.The y-axis represents the home range size in cm². The x-axis represents different treatment levels (Control, Low, and High). Left panel: Non reproductive individuals. Right panel: Reproductive individuals. The whiskers extend to the most extreme data points not considered outliers. Males (blue) and female (pink) subjects.

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Shell selection

Based on the results of my analysis, the sex of the individuals (GLMM, est. ± SE = -0.020 ± 0.021, z = -0.096, p = 0.924) didn't show a significant influence the estimated variable for the shell selection. Additionally, the treatment conditions, both low (GLMM, est. ± SE = -0.105 ± 0.237, z = -0.444, p = 0.657) and high (GLMM, est. ± SE = 0.302 ± 0.241, z = 1.254, p = 0.210), did not significantly affect the outcome. However, the treatment in the high condition indicated a potential yet non-significant trend. (Figure 13).

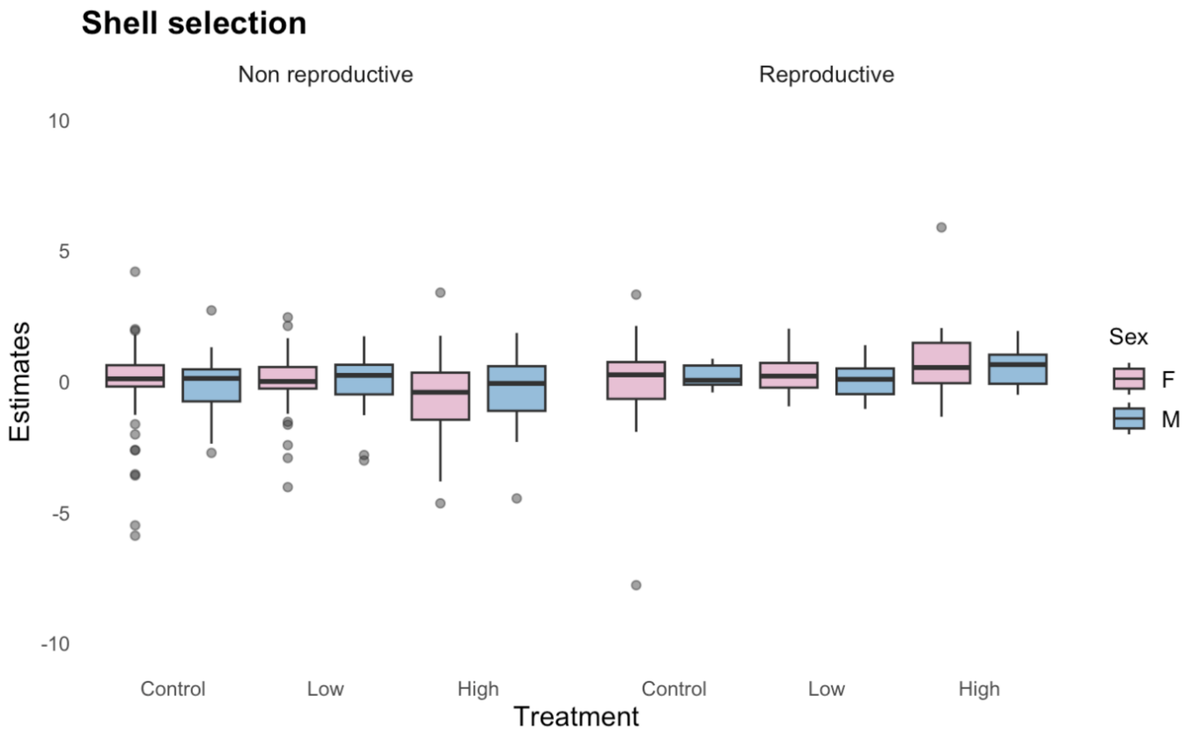

Figure 9. Shell selection for in oxazepam treatments (Control, Low, High) for males (blue) and females (pink) individuals, separated into non-reproductive and reproductive groups. Positive values indicate attraction to the shell. Negative values indicate repulsion from the shell. The whiskers extend to the most extreme data points not considered outliers, and the points beyond the whiskers are outliers.

4. Discussion

Pharmaceuticals that enter the environment have been identified as ecological change drivers and it is frequently shown that psychotropic medications (like oxazepam) cause behavioural changes in fish and other aquatic invertebrates (Buřič et. al., 2018; Brodin et al., 2013). Fish characteristics like swimming activity, aggression, feeding rate, and boldness can be impacted by the pollution caused by medications used to treat anxiety disorders (Brodin et al., 2014). The pharmaceutical we used in this experiment is Oxazepam, classified as a benzodiazepine anxiolytic. We chose oxazepam because it is widely prescribed for the management of conditions such as anxiety, sleep disorders, and muscle spasms. It is also widely found in aquatic ecosystems, and it has also been shown to affect animals. But what are the effects on reproductive endpoints? That's the question that I wanted to answer with this thesis. All the above led me to the belief that oxazepam will possibly affect reproduction in aquatic species, therefore I conducted research on how in *Neolamprologus multifasciatus*.

4.1 Differences between individuals in the control treatments.

Differences between reproductive and non- reproductive individuals

Reproductive males showed notable increase in aggression (both attack and display), in comparison with the non-reproductive ones. Reproductive females showed more submission than the non-reproductive ones, which agrees with findings from previous studies (Bose et. al., 2021), potentially relating to parental care. I had predicted that the individuals that were reproductively successful would use more space in the tank, expecting that the dominant individuals are the ones that reproduce, hence they control the resources. I based my assumption on previous researches that suggested that territory sizes seem to significantly differ between sexes and reproductive-non reproductive individuals in the wild (Bose et al., 2021). The results were opposite than my hypothesis. Reproductive individuals had smaller home ranges compared to non-reproductive ones, likely due to the need for territory defence and parental care. Reproductive males on the contrary had bigger home ranges than reproductive females. Possibly because males can reproduce with females from different sub-territories (Bose et al., 2022a).

Differences between sexes

Regarding the sex specific behaviours, it seems that males in our study are significantly more aggressive than the females and the females are significantly more submissive than the males. That can be explained with the fact that males show dominant- submissive behaviours, hence females are more equally distributed in terms of status (Bose et al., 2022 . Also, females can show submission to males in periods of parental care (Bose et al., 2022). Some previous finding about aggressiveness that have been recorded in N. multifasciatus (Schradin and Lamprecht,2000) found that groups with dominant males exhibited lower levels of female-to-female aggression because the males could mediate conflicts among the females in a laboratory environment. Furthermore, Gübel et al. ,2021 observed that when a foreign female has the chance to join the group, males become more aggressive towards their own females.

4.2 Oxazepam alterations on the measured endpoints.

My findings show that oxazepam exposure indeed can influence both reproductive success and social behaviours. My initial hypothesis was that oxazepam is expected to reduce aggression, therefore, more individuals within oxazepam exposed groups will be able to reproduce compared to the ones that weren't exposed. I expected that the aggressiveness in dominant males will be reduced, hence subordinate males will have higher chances in reproduction. Previous findings by Kohler, 1998 and Bose et al. 2022 suggested that reproduction is shared mostly among dominant males in *Neolamprologus multifasciatus* and the ownership of gastropod shells is essential to both survival and reproduction in the wild (Kohler, 1998; Bose et al. 2022). Also, in brown trout populations, when oxazepam is added to groups with dominant and subordinate members, results increase in the boldness of subordinates (McCallum et al., 2021). Indeed, high doses of oxazepam significantly increased reproductive success in males. In particular, it allowed smaller males to become reproductively successful, whereas they were unable to reproduce under control conditions. However, this pattern was less pronounced in females, which is also consistent with findings about multis reproduction from the wild (Williams, 2004). Oxazepam did not seem to affect the production of offspring but did affect the division of parentage.

In my findings, display (aggression) behaviours in high doses of oxazepam was significantly reduced in both reproductive and non-reproductive individuals. This result potentially reflects the previous findings about more even distribution of the reproduction among males. Former studies on wild roaches (*Rutilus rutilus*) treated to high oxazepam concentrations (280 µg/L) found that the roaches became bolder while, at low treatment (0.84 µg/L) boldness and activity increased compared to control treatment (Brodin et al., 2017). Other studies failed to detect any behavioural effects of oxazepam in fish species *Pimephales promelas* (Huerta et al.,2016).

Previous findings suggested that oxazepam significantly reduced the travelled distance and the activity of crayfish (*Procambarus virginalis*) in reproductive individuals compared to non-reproductive individuals (Kubec et al.,2019). In our research neither low nor high treatment suggested alterations in home range size compared to control. This suggests that while oxazepam affects specific social behaviours, it may not drastically change overall spatial dynamics and resource use, but engage in fewer aggressive behaviours with one another. For the shell selection function, we didn't find a significant effect of sex, the oxazepam treatment, or reproductive success. This might be explained from the fact that there were space constraints in the tank or due to their strong reliance on shells (Schradin & Lamprecht, 2000), it may be particularly challenging for these fish to leave their shells regardless of the exposure condition. In any case I would suggest further investigation in the future.

Future Directions

Future research should consider show how early life exposure will affect the later life of individuals. That can be by repeat the experiment but start it since the offspring stage. extending the duration of experiments to observe the potential effect of oxazepam exposure in selected lines. Investigating these intergenerational effects and the influence of environmental stressors is crucial for understanding the full evolutionary impact of pharmaceutical contaminants.

Even though, the laboratory environment in our study was highly naturalistic (natural resources and social environments), we might detect additional effects of oxazepam that could have selective consequences, when additional ecological factors are considered. This can include predator treatments, food competition, heterospecific competitors, etc. Thus, a comprehensive understanding of oxazepam's impacts necessitates further research incorporating these additional ecological variables to fully grasp its potential selective consequences in natural settings of *Neolamprologus multifasciatus*.

Conclusion

To sum up, high treatments of Oxazepam can affect the probability of reproduction in males and also reduce aggression. Such changes might have significant consequences for natural selection (Sober, 2014). Environment-adapted individuals have a higher chance of surviving, dispersing the genes that contributed to their success, and passing them to the next generation (Sober, 2014). When smaller males can reproduce under high treatment exposure, this has implications for what traits can be favoured or disfavoured by selection in the wild, potentially disrupting established social systems (Brodin et al., 2014). Territory sizes seem to significantly differ between sexes and reproductive versus non-reproductive individuals in our experiment. We found that reproductive individuals had smaller home ranges compared to non-reproductive ones, likely due to the need for territory defence and parental care. Additionally, reproductive males had bigger home ranges than reproductive females, possibly because males can reproduce with females from different sub-territories (Bose et al., 2022a). We did not find an effect of oxazepam on territory size, suggesting that while oxazepam affects specific social behaviours, it may not drastically change overall spatial dynamics. These findings provide insights into sex-specific reproductive behavioural differences, which can help us understand the dynamics within multi-population systems. The real important takeaway is that exposure to pharmaceuticals can have sublethal effects on behaviour that may cascade to affect reproductive success. As pharmaceuticals continue to be widespread environmental contaminants, understanding their ecological consequences and relevance to evolutionary selection is crucial for protecting aquatic ecosystems.

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Appendix 1

This appendix contains a summary written by the Paul Nuehrenberg, explaining how the classifier works. This summary is included here for additional context and clarification. It is important to note that this summary was not part of this thesis:

An automated approach was used to detect and classify social behaviors among interacting individuals. An XGBoost model was trained to distinguish seven behaviors (approach, lateral display, frontal display, dart/bite, chase, body quiver, hidden in shell) from each other and from the background category (no social behavior). This model utilizes distributed gradient-boosted decision trees, which are well-suited for classification and regression tasks with structured data. The parameters of these decision trees are tuned while fitting the model to data. For this task, the output probabilities for each behavioral category were optimized to fit the labeled training data, which consisted of approximately 300,000 time points (i.e., video frames) where the behavioral category between two interacting fish was known. To compile this training dataset, 9 representative videos featuring 15 fish (approximately 10 minutes per video) were annotated using BORIS, a software for behavioral observations from video data. Behavioral observations (i.e., intervals with start and stop timestamps) were then sampled at each time point (at a temporal resolution of 30Hz, matching the video frame rate), and multiple kinematic and postural metrics were calculated from the tracking data (head, center, and tail video coordinates for each fish) for three time windows (0.5s, 1.0s, 3.0s). These metrics provide a detailed description of dyadic fish interactions and served as the basis for the classification task.

The model was further trained using a supervised active learning pipeline, where it was enhanced over multiple iterations by explicitly including false positives and corrected, previously misidentified behaviors into the training dataset. The final training dataset included approximately 1,200 behaviors. A stratified k-fold cross-validation technique (5 folds of the dataset, stratified by individuals) was used, allowing for the validation of the model on an independent test dataset in each training round (20% of individuals were randomly assigned to this test dataset for each fold). Once the model's accuracy was deemed suitable for the classification task, it was retrained on the full dataset, and the resulting model was used for predictions on the entire set of trials.

Additionally, a post-processing scheme was implemented to identify optimal thresholds for the average and maximum detection probability and duration of behavioral detections for each category. A grid search was conducted over these parameters (thresholds for minimal duration between 0s and 2s, for minimal average probability between 0.0 and 0.9, and for minimal maximum probability between 0.0 and 0.9), optimizing the resulting relative likelihood of a simple linear regression model (true dyadic behavior durations for each versus predicted dyadic behavior durations) via Akaike weights. The optimal thresholds were then applied to discard behavioral detections that did not meet these criteria.

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