How is Parental Care Impacted by Pharmaceutical Pollution in *Neolamprologus multifasciatus*?

Behavioural and reproductive responses to diazepam exposure in a shell-dwelling cichlid

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Abstract

Pharmaceutical pollutants are increasingly recognized as a threat to aquatic ecosystems due to their potential to alter animal behaviour. This experiment investigated the effects of the anxiolytic drug diazepam exposure on reproduction and parental care in the social cichlid *Neolamprologus multifasciatus*. Using replicate aquariums stocked with males and females, the fish were exposed to either a control concentration, a low treatment at 1 µg/L, or a high treatment at 50 µg/L of diazepam in a laboratory setting for 96 days. I monitored which females reproduced over this time, and quantified maternal aggression using mirror trials. The proportion of females that produced fry did not differ between diazepam treatments, although the number of fry that they produced was higher in the high compared to low treatment. I found that diazepam exposure significantly reduced display aggression at high doses by mothers in response to a presented mirror, but had no effect on overt aggression. Additionally, females in the high treatment swam in front of the mirror more times and emerged faster from their shelter shells, suggesting increased activity or exploratory behaviour. These findings indicate that diazepam alters parental behaviours, and also significantly impacts reproductive output, highlighting the importance of including behavioural endpoints in ecotoxicological assessments.

Keywords: Pharmaceutical pollution, Diazepam, Parental care, Reproduction, Cichlid fish, *Neolamprologus multifasciatus*, Mirror trial, Ecotoxicology.

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1. Introduction

Pollution from pharmaceuticals and personal care products (PPCPs) is a rising global concern, due to their increasing detection in aquatic ecosystems and their potential to affect non-target organisms (Boxall et al. 2012; Wilkinson et al. 2022). PPCPs enter waterways through different pathways, with one example being wastewater, particularly because conventional wastewater treatment plants often fail to completely remove biologically active compounds (Brodin et al. 2014; Wilkinson et al. 2022). Aquatic environments are vulnerable to these inputs, as resident organisms are continuously exposed to wastewater-borne substances both via the water column (bioconcentration) and their food (bioaccumulation) (Boxall et al. 2012; Brodin et al. 2014; Lorenzi et al. 2014). PPCPs, including psychoactive pharmaceuticals such as benzodiazepines, act on evolutionary conserved targets such as GABA (gamma-aminobutyric acid) receptor, raising concern about their unintended effects across taxa (Boxall et al. 2012). Despite growing awareness, behavioural endpoints remain underrepresented in ecotoxicological research, even though they may serve as early-warning signals for sublethal disruption (McCallum et al. 2021).

Among the most frequently detected PPCPs in aquatic systems are benzodiazepines, a class of psychoactive pharmaceuticals. Diazepam, commonly known by the trade name Valium^{TM}, is a widely prescribed benzodiazepine used to treat anxiety, insomnia, muscle spasms and seizures (Fick et al. 2017; National Library of Medicine, 2023). Benzodiazepines have been detected in over 80 % of the largest European river systems, with total median and mean concentrations of 5.4 and 9.6 ng/L^{-1} respectively, and maximum concentrations near ~1 µg/L (Fick et al. 2017; Wilkinson et al. 2022). Another benzodiazepine oxazepam was found to remain biologically active for decades in the sediment (Klaminder et al. 2015), and compounds like this are considered pseudo-persistent pollutants because they are continually being added to the environment via wastewaters (Wilkinson et al. 2022). Diazepam works by enhancing the activity of the inhibitory neurotransmitter GABA, producing anxiolytic effects (National Library of Medicine, 2023). Although designed to target human physiology, diazepam can also affect aquatic organisms because they similarly possess GABA receptors (Renier et al. 2007). Previous research has found that exposure to benzodiazepines can alter important behaviours such as aggression and boldness in fish (Brodin et al. 2014; McCallum et al. 2021). Behavioural alterations in fish have been observed at concentrations of 1 µg/L (Brodin et al. 2013). Given that benzodiazepines are widespread in many surface waters, and their behavioural altering potential in vertebrates, we need a clearer understanding of how exposure

to these compounds affects animal behaviours important for their survival and reproduction.

Parental care is vital for the successful rearing of offspring and plays a central role in determining offspring survival in many animal species, including many fishes (Royale et al. 2012). Since reproduction is a major component of fitness, an organism's ability to pass on its genetic material relative to others in the population, therefore alterations in parental behaviour may negatively affect fitness (Campbell et al. 2021). In fishes, parental care strategies vary widely, from hiding or guarding eggs to a direct role where they actively defend offspring (Zimmermann et al. 2021; Royle et al. 2012). If pharmaceuticals like diazepam alter the ability of parents to perform aggression or perceive threats in their environment, exposure may therefore impair a parent's ability to defend and care for their offspring. Studies have shown that anxiolytics can suppress aggression (McCallum et al. 2021), their effects on maternal aggression specifically, in context of offspring protection and parental care, remain poorly understood.

In this experiment, I investigated how exposure to the anxiolytic medication diazepam impacts reproduction and parental care of the cichlid *Neolamprologus multifasciatus*. This group-living fish is endemic to Lake Tanganyika, East Africa, and exhibits complex social behaviour. *N. multifasciatus* live in mixed sex groups (group sizes range from 2-20) and their survival and reproduction in the wild depends on securing a place within one of these social groups and acquiring suitable shelters (Bose et al. 2022). As one of the Lamprologine cichlids, they specifically utilize empty gastropod (*Neothauma Tanganyicense*) shells (Bose et al. 2021). Females lay their eggs and rear offspring inside these shells, providing parental care by guarding the shell and emerging fry, especially to deter intruders from approaching too closely (Schradin & Lamprecht. 2002). Previous studies show that female-female aggression increases when fry is present (Bose et al. 2022), however this is rarely studied under the effects of anxiolytics. Diazepam's therapeutic effect reduces stress and anxiety (Pritchett et al. 1989), which may allow individuals that are normally excluded from reproducing (e.g., due to high levels of social stress or aggression), to engage in reproductive behaviours (Bose et al. in prep.). Therefore, diazepam could potentially increase the number of reproductively active individuals within a social group, making it important to investigate both behavioural and reproductive endpoints in exposed populations.

In this experiment, I aimed to investigate how exposure to the anxiolytic pharmaceutical diazepam influences reproduction and parental care in the shell-dwelling cichlid *N. multifasciatus* by addressing two main research questions: Does pharmaceutical exposure to diazepam influence reproduction and reproductive timing in *N. multifasciatus* females? Second, does diazepam affect

maternal aggression? To address these questions, I exposed social groups of *N. multifasciatus* to different concentrations of diazepam and, assessed female reproduction and aggression (both with and without fry) in response to a presented mirror. I hypothesised that exposure to diazepam would allow more females to become reproductive, that diazepam would dampen aggression towards a mirror, and that females with offspring would be more aggressive than those without offspring.

2. Materials and methods

2.1 Neolamprologus multifasciatus

Neolamprologus multifasciatus social groups typically consist of a dominant male, subordinate males, several reproductive females, and juveniles. Each individual maintains a home shell, which it regularly returns to and defends, and guards a small sub-territory within their group's broader territory (Bose et al. 2021; Jordan & Lein 2021; Schradin & Lamprecht 2002). Females lay eggs (that they attach to the inner wall of the shell) and care for offspring within these shells, with their sub-territories containing one to five shells (Schradin & Lamprecht 2002). Females provide parental care for fry until they can occupy a shell of their own, usually when fry reach 10 mm standard length (Bose et al. 2022; Schradin & Lamprecht 2002). Multiple females breed simultaneously in their social groups while only the large dominant male within each group breeds (Bose et al. 2022). *N. multifasciatus* display a range of behaviours, including aggression as well as submission. Males tend to direct aggression towards other males, while females are more aggressive towards other females (Gübel et al. 2021; Schradin & Lamprecht 2002).

For this experiment, adult *N. multifasciatus* were purchased from multiple aquarium retailers across the EU to ensure genetic diversity. The fish came from Hageby Cikliden and Zoo.se in Sweden, Alex Tropicals in Czech Republic, and Aquarium Glaser GmbH in Germany.

2.2 Experiment setup

This experiment began on 23 January 2025 (day 1) with permit (Dnr A-5-2023) from Jordbruksverket. Fish were assigned to 21 tanks, each measuring 50 x 70 x 40 cm (approximately 115 L), four females and three males were assigned to every tank. Before being placed in the tanks, each fish was tagged for individual identification, with visible implant elastomer tag (Northwest Marine Technologies). Each tank was equipped with a recirculating biological filter (Aquaclear 70, with no carbon insert), an air stone, and an aquarium heater (Eheim Thermocontrol 100 W). The tanks were filled with 5 cm of cichlid sand (Aqudeco Cichlid sand) and given 24 *N. Tanganyicense* gastropod shells to provide shelter and breeding chambers (ten additional shells were added to each tank on 7 March 2025). Fish were fed with Dr. Basseeler BioFish food (Aquarium Münster) and monitored daily for health and mortality. Water quality was routinely measured in three randomly selected tanks per treatment every other week, or any time a mortality had occurred. We monitored: Nitrate, nitrite,

general hardness (GH), carbonate hardness (KH), and chlorine levels were measured using the JBL ProAquaTest Easy 7-in1 (Appendix A, Table A1). Dissolved oxygen and water temperature were recorded using a YSI Ecosense ODO200 optical dissolved oxygen meter. pH was measured using a Pocket Pro+ Multi 2 tester with a replaceable sensor. The light followed the natural photoperiod with approximately 12 hours of light and 12 hours of darkness, to mimic a natural day-night cycle in Lake Tanganyika to maintain typical behavioural rhythms in the fish.

Fish were divided into a control treatment (0 µg/L diazepam), and two treatment groups (a low and a high concentration), each group consisting of seven replicate tanks (21 tanks total). We exposed fish to diazepam (Diazedor, Salfarm Scandinavia – a liquid solution of diazepam) by spiking the aquarium water with diazepam to reach the desired exposure concentrations. These included a low treatment (2 µg/L diazepam), representing concentrations that may occur near wastewater discharge points, and a high treatment (50 µg/L diazepam), which served as a positive control well below lethal levels for fish (Straub et al. 2008) Control tanks were spiked with the same volume of ethanol used in the treatment tanks but with no drug added. Every four weeks, 25 % of the water was replaced in each tank. The new 25 % was re-dosed with diazepam to match the target concentrations (i.e., 1 µg/L or 50 µg/L), while the remaining 75 % of tank water was re-dosed at 30 % of the target concentrations. This approach accounted for expected losses due to degradation and uptake over time. The 30 % re-dosing rate was based on prior stability analysis (not part of this thesis).

In this experiment, we collected water samples for chemical analysis to monitor the actual exposure levels. The preparation and analysis of these samples was not a part of this thesis. Briefly, the procedure involved collecting 12 mL of tank water, filtering 5 mL through a 45 µm filter, and adding an internal standard prior to analysis by liquid chromatography mass spectrometry. The average measured concentrations of diazepam in the low treatment tanks were 1.37 ± 0.43 µg/L (n = 8 samples) and high treatment 37.68 ± 1.78 µg/L (n = 3 samples). It should be noted that only a small subset of samples was analysed for this thesis, and additional analyses will be conducted in the future.

2.3 Reproductive and Behavioural Assessment

I tracked fish reproduction across the experiment using regular observations and measured maternal aggression using mirror trials. Both are described in turn below.

2.3.1 Fry observations

Fry observations began on day 20 of the experiment, and the first fry were detected on day 24 (Fig 1). Fry observations continued until day 96 and initially, I did more frequent fry observations in the beginning and less frequent as the experiment progressed. (n = 36 fry observation days in total). All observations were conducted at approximately the same time each day, in the morning, prior to feeding. Each tank was observed five minutes. During these sessions, I recorded whether each female had fry swimming in and around her shell. When possible, the number of fry of each female was counted (only possible when they grow large enough to emerge from the shell entirely).

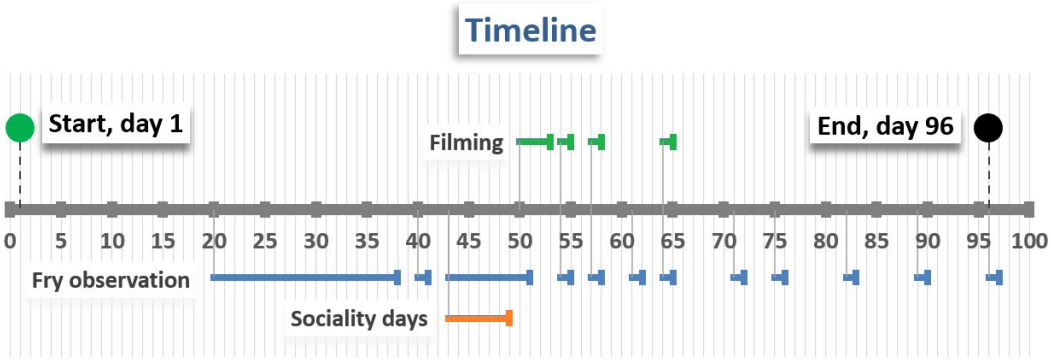

Figure 1. Experiment timeline. The experiment stared at day 1, with fry observations (blue) beginning on day 20 and continuing until day 96 (n = 36 days). Sociality days (orange) refer to seven days during which another master's student conducted fry observations on four tanks per day as a part of a separate project. Filming (green) were conducted on six occasions, starting at day 50.

2.3.2 Maternal aggression

Assessments of maternal aggression were conducted using mirror trials between day 50 and day 64 (Fig. 1). I used mirror trial assays which provide a method for measuring aggression in fish without introducing variation from a live opponent. Although mirror assays do not fully mimic the complexity of social contests, they offer a reliable proxy for aggressivity (McCallum et al. 2017).

Mirror trials were conducted immediately after any day's fry observations. For each female with fry, a corresponding female without fry was also selected for a mirror trial. When possible, the female without fry was selected from the same tank or, if not available, from a tank within the same treatment. A mirror (14.5 x 8.9 cm) was placed inside the tank 5 cm (approx. one shell-width) away from the shell that the focal female was occupying (Fig. 2). Filming of the trials was conducted top-down using a GoPro Hero 10 black with the following settings: narrow field of view, 30 frames per second (fps) and 4K resolution. When mirrors were placed next to the females, they would quickly hide inside their shells. Thus,

I was able to quantify the latency for the fish to emerge from their shells during the recordings. I also quantified the time fish spent in front of the mirror (regardless of whether they were performing behaviours) based on outlining a so called mirror zone (5 x 8.9 cm) (Fig. 2).

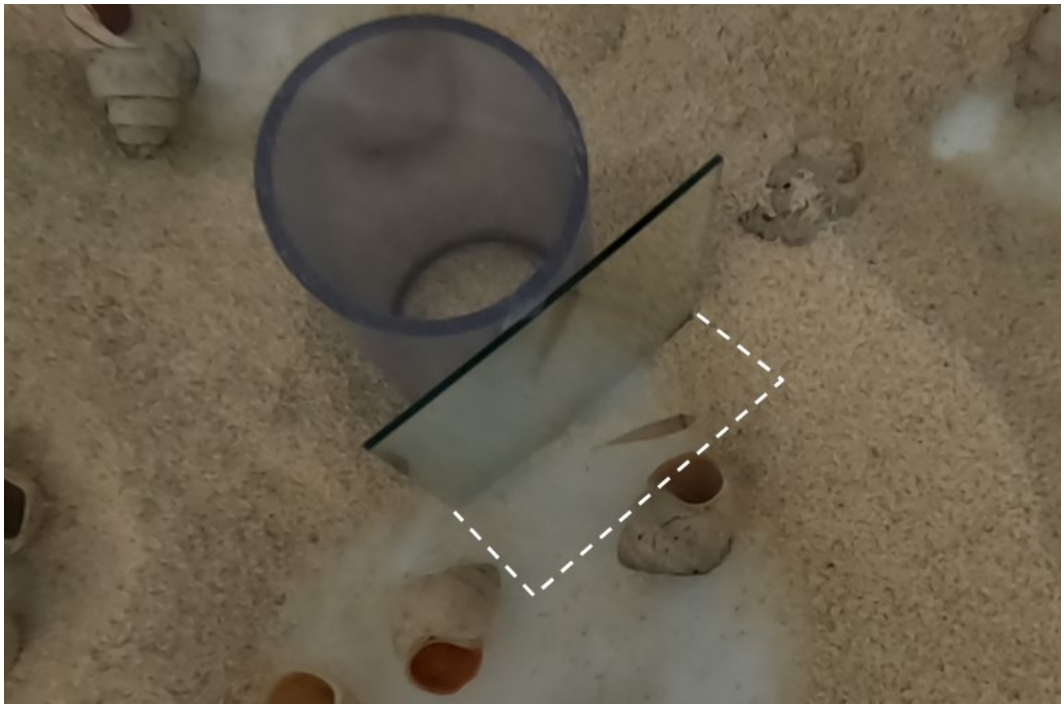

Figure 2. Screenshot from behavioural recording video. The focal female is positioned in the mirror zone (dashed white lines). The focal females home shell is located directly in front of the mirror with opening towards mirror.

All videos were analysed using the software BORIS (v. 9.0.8; BORIS 2025), with behaviours scored either as state behaviours (as a time duration variable) or as a discrete behaviour (as a count variable), based on a predefined ethogram (Table 1; Bose et al. 2023). Videos were scored blind to treatment and fry presence. For each trial, exactly ten minutes were analysed starting from the moment the female emerged from her shell. Female body length was measured from screenshots of each female positioned next to the mirror with known dimensions. Standard length of each female was measured ImageJ (Schneider et al. 2012).

Table 1. Ethogram used for behaviour scoring of N. multifasciatus.

Behaviour	Description	Scored as
Display aggression		
Frontal display	Focal fish face the opponent head–on, often with an erect posture and flared fins.	State event
Lateral display	Focal fish presents its side to the opponent often with a rigid body and flared fins.	State event

Overt aggression		
Mirror push	Focal fish makes contact with the mirror by repeatedly pushing its head against the mirror.	State event
Bite/ram	Focal fish swims quickly towards opponent, sometimes making contact.	Point event
Other		
Shell hiding	Focal fish hides in their home shell.	State event
Sand spitting	Focal fish collects sand in its mouth and spits it out, often towards other fish/territories.	Point event
Head shake	Focal fish shakes its head.	
Mirror zone	Focal fish spend time in designated mirror zone, within 5 cm in front of the mirror.	State event
Behind mirror	Focal fish spend time behind mirror, often interacting with its reflection in the plastic tube.	State event
Disturb	Focal fish gets disturb by other fish, preventing it from continuing behaviour.	State event

For statistical analysis, aggressive behaviours were grouped into two categories: display aggression or overt aggression. Display aggression included frontal display and lateral display, which are non–contact visual signals typically used in territorial or social contests. Overt aggression included bite/ram and mirror push, which often involve physical contact or attempt thereof.

One female was excluded from the behavioural dataset due to a trial where she, directly after emerging, was highly disturbed by another fish and fled out of filming range and did not re–emerge into view during filming. This reduced sample size to 58 females, control (with fry = 9, without fry = 9, n = 18), low (with fry = 10, without fry = 10, n = 20) and high (with fry = 10, without fry = 10, n = 20).

2.4 Statistical Analyses

All statistical analyses were conducted in R (v.4.2.3; R Core Team 2023) with packages glmmTMB (Brooks et al. 2017), *emmeans* (Lenth, 2025), performance (Lüdecke et al. 2021), DHARMa (Hartig, 2024), tidyverse (Wickham et al. 2019), patchwork (Pedersen, 2024) and TMB (Kristensen et al. 2016).

Model assumptions were evaluated using simulation-based residual checks (DHARMa), Shapiro-Wilk tests, and diagnostic plots (performance). Model

selection was guided by Akaike's Information Criterion (AIC) in which interaction terms were assessed and retained in the final model only when they significantly ($P = 0.05$) improved model fit based on likelihood ratio tests (*drop1*). Post hoc comparisons were conducted using the *emmeans* package for pairwise differences and *emtrends* for estimated trends over time.

2.4.1 Reproduction statistics

To assess the potential effects of diazepam exposure on reproduction and number of offspring produced, three analyses were conducted:

1) The daily proportion of females with fry swimming by their shell entrance.
2) The daily number of fry observed in each tank.
3) The day when females were first seen with offspring.

For analysis 1 and 2, binomial Generalized Linear Mixed Models (GLMMs) were used, with the number of females observed with fry or the number of fry over the number of live females as the response variable (i.e., successes/total). Treatment (control, low, high), day (observation day), and their interaction term were included as fixed effects, with tank ID as a random intercept (Appendix A, Table A2). Post hoc tests of trends over time were assessed using *emtrends*.

For analysis 3, a two–step hurdle model analysis was used. First, a binomial GLMM assessed whether treatment affected whether or not each female ever produced fry during the observation period. The response variable was binary (yes/no), with treatment as a fixed effect and tank ID as random intercept. The second analysis included only females that produced fry, and I analysed the day when fry was first observed with each female using a Gaussian GLMM. This model included treatment as a fixed effect and tank ID as a random intercept.

2.4.2 Behavioural statistics

To assess the behavioural responses to diazepam, four analysis were conducted:

1) Total duration of display aggression (seconds during which the focal fish engaged in either frontal or lateral displays).
2) Total count of overt aggression (number of bite/ram and mirror push events combined).
3) Total time spent in the mirror zone and number of entries into the mirror zone.
4) Latency (in sec) to exit the home shell after mirror placement.

Each behaviour was analysed using GLMMs. Count data (e.g., overt aggression and mirror visits) were modelled using a negative binomial distribution to account for overdispersion. Duration data (e.g. display aggression and mirror zone duration) were modelled using Gaussian distributions. Latency data were log-transformed to improve residual normality. All models included treatment (control, low, high), fry presence (yes/no), and body length as fixed effects, with tank ID as a random intercept. For the behaviour data the interaction between treatment and fry presence never improved model fit and was omitted from the final model (Appendix A, Table A3).

To account for variation in actual observation time which the focal females could interact with the mirror, I included an offset term in the models. This offset was calculated as the time duration when the focal fish had the opportunity to interact with their mirror reflection, by taking the observation time and subtracting the fish's time to emergence, and any time durations when the fish was behind the mirror or physically interacted with by another tank mate.

3. Results

3.1 Reproductive outcomes

I detected a strong effect of day (estimate ± SE = 0.033 ± 0.0050, z = 10.22, P = < 0.0001), indicating that the number of females with fry increased over time, regardless of treatment (n = 538; Fig. 3a). Post hoc comparisons showed that the number of females that reproduced did not differ with treatment (P > 0.19) (Appendix B, Table B1). Again, I detected a strong effect of day (estimate ± SE = 0.036 ± 0.0042, z = 8.59, P = < 0.0001), indicating that the number of fry increased over time, regardless of treatments (n = 547, Fig. 3b). I found a significant difference in the rate of increase: fry numbers increased more rapidly in high treatment than in low treatment (estimate ± SE = 0.019 ± 0.0066, z = –2.82, P = 0.013) (Appendix B, Table B1). However, the difference between the control treatment and low treatment (P = 0.39) and the difference between control treatment and high treatment (P = 0.21) was not significant (Appendix B, Table B1).

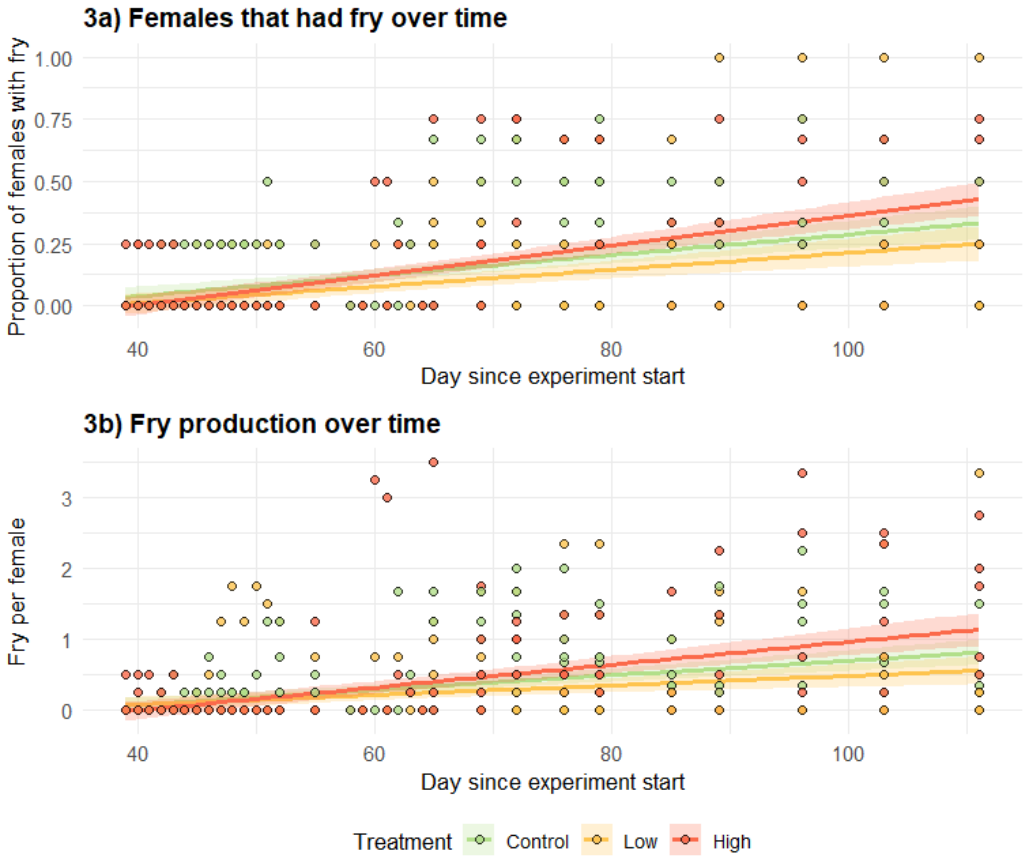

Figure 3. Reproductive outcomes by treatment (control = green, low = yellow, high = red). a) Proportion of females with visible fry over time. b) Fry production over time,

calculated as fry per female. Lines indicate fitted means with ribbons indicating 95 % confidence intervals.

I analysed whether treatment affected the timing of when females had fry with a binomial mixed-effects model (n = 48, Fig 4). I did not detect any significant effects of treatment on when fry was first observed (P > 0.62). On average, females with fry were observed at day 46.0 ± 4.22 in control, 52.3 ± 5.10 in low treatment and 51.9 ± 4.60 in high treatment (Appendix B, Table B1).

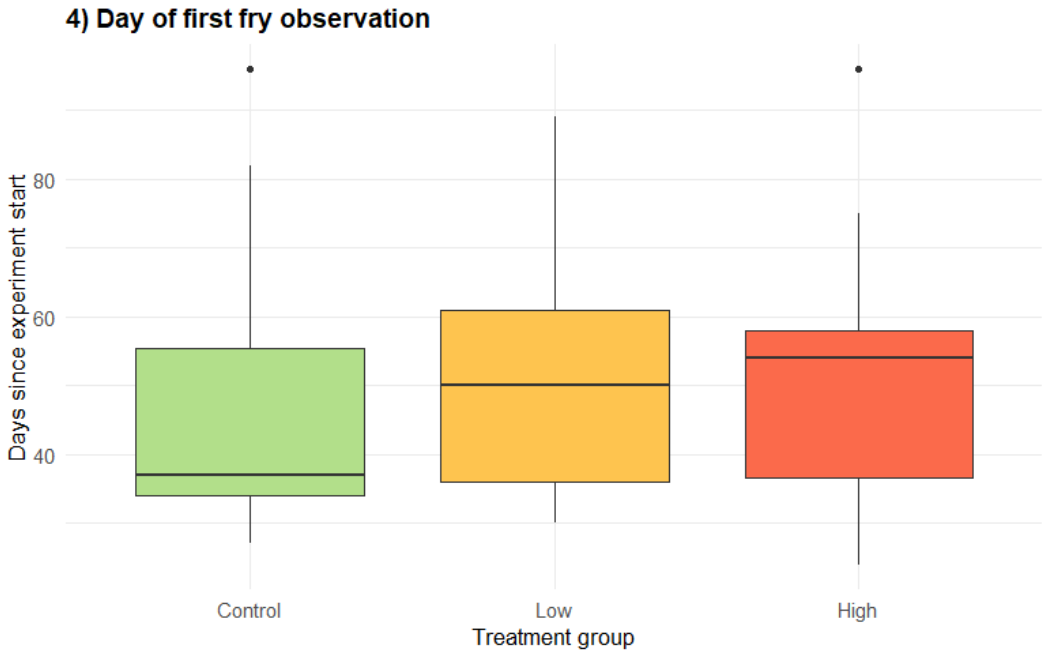

Figure 4. Day of first fry observation per treatment group (control = green, low = yellow, high = red). Boxes show the number of days since start of the experiment when fry were observed by a female's shell. Boxplots represent interquartile ranges and horizontal lines indicate medias.

By the end of fry observation period (day 96), I recorded that 64.10 % of the females had fry some point during the observation period: 80 % in the control treatment (20 out of 25), 50 % in the low treatment (13 out of 26), and 63 % in the high treatment (17 out of 27). Six out of 84 females died during the experiment: three in control, two in low, and one in high. No fry was detected in two tanks: one control tank and one low treatment tank.

3.2 Maternal aggression

I detected a weak, but not statistically significant, effect of fry presence on female display aggression towards the mirror: females with fry tended to display aggression for longer durations compared to those without fry (estimate ± SE = 55.09 ± 30.79 sec, z = 1.79, P = 0.074) (n = 58; Fig. 5a). I found no significant effects of body length on display aggression towards the mirror (P = 0.27)

(Appendix C, Table C1). Post hoc comparisons revealed that fish in the low treatment group displayed significantly longer than those in the high treatment (estimate ± SE = 95.6 ± 36.9 sec, t = 2.59, $P = 0.033$). No other pairwise comparisons were significant ($P > 0.33$) (Table B2). Females with fry in the control treatment displayed the longest (mean = 248.52 sec, $n = 8$, SD = 150.49), while females without fry in the high treatment displayed the shortest on average (mean = 118.032 sec, $n = 9$, SD = 115.44) (Appendix C, Table C2).

I did not detect any significant effects of presence of fry ($P = 0.28$) nor of body length ($P = 0.50$) on overt aggression towards the mirror ($n = 58$; Fig. 5b; Appendix C, Table C3). Post hoc comparisons between treatments showed no significant differences between any treatment ($P > 0.54$) (Appendix C, Table C3). Overall, overt aggression levels remained consistently low across treatments, with no apparent treatment-related patterns (Fig. 5b). Females without fry in the low treatment group displayed the highest average number of overt aggressive acts (mean = 6.5, $n = 4$, SD = 4.20), while females with fry in the high treatment group showed the lowest average (mean = 2.0, $n = 5$, SD = 1.00) (Appendix C, Table C2).

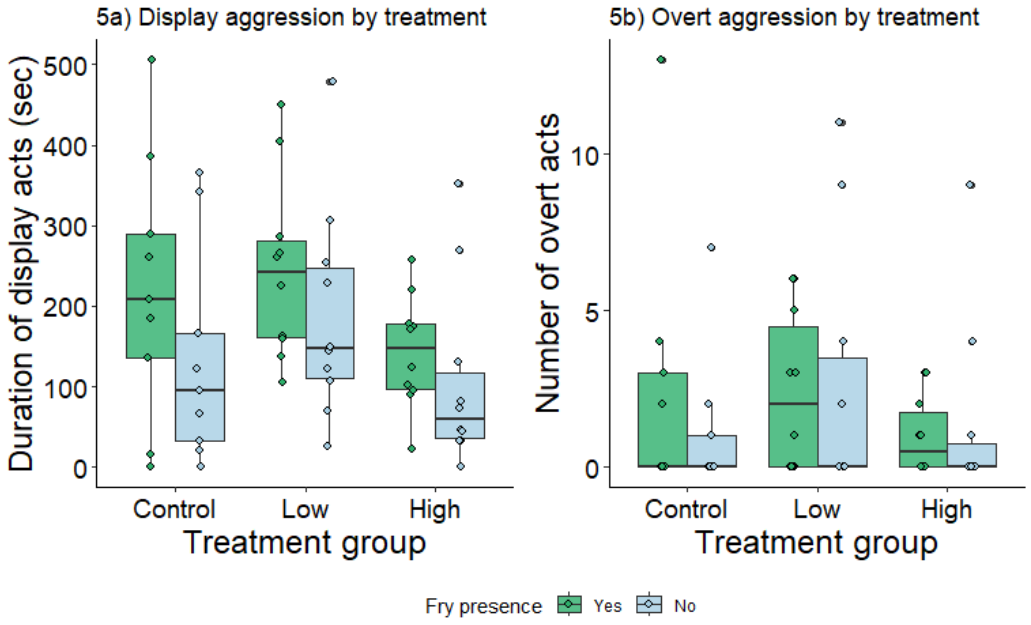

Figure 5. Display aggression and overt aggression by treatment (control, low and high) and fry presence (yes = green; no = blue). Boxes represent interquartile ranges, with horizontal lines indicating medians and individual data points jittered for clarity. a) Display aggression duration (sec) across treatment and fry presence. b) Overt aggression counts across treatment and fry presence.

During the behavioural trials, I observed that in 49 out of 58 recordings, at least one other fish besides the focal fish entered the mirror zone. Only one focal

female exhibited sand-spitting behaviour, which she performed five times. None of the females displayed head-shaking behaviour. Females also visited their home shell, nine females in the control treatment, 13 females in low treatment and 14 in high treatment (Appendix C, Table C4). Females without fry in the low treatment spent the longest time hiding in their shell on average during trials (mean = 54.60, n = 5, SD = 58.79), while females with fry in the control treatment spent the shortest time hiding (mean = 10.88, n = 4, SD = 8.10).

3.3 Time to emerge

I detected no significant effects of fry presence (P = 0.44) on the time to emerge from their shell (n = 58; Fig. 6; Appendix C, Table C5). Estimated marginal means showed that fish in high treatment emerged significantly faster than those in the control treatment (P = 0.032), while the difference between low treatment and high treatment (P = 0.12) and the difference between control and low treatment (P = 0.80) were not significant (Appendix C, Table C2). On average, females in the control treatment emerged after 90.23 seconds when fry were present and after 152.57 seconds when no fry were present. In the low treatment, females with fry emerged after 118.17 seconds, while those without fry emerged faster, after 64.97 seconds. In the high treatment, females with fry emerged the fastest, after 29.95 seconds, compared to 54.43 seconds for females without fry (Appendix C; Table C2).

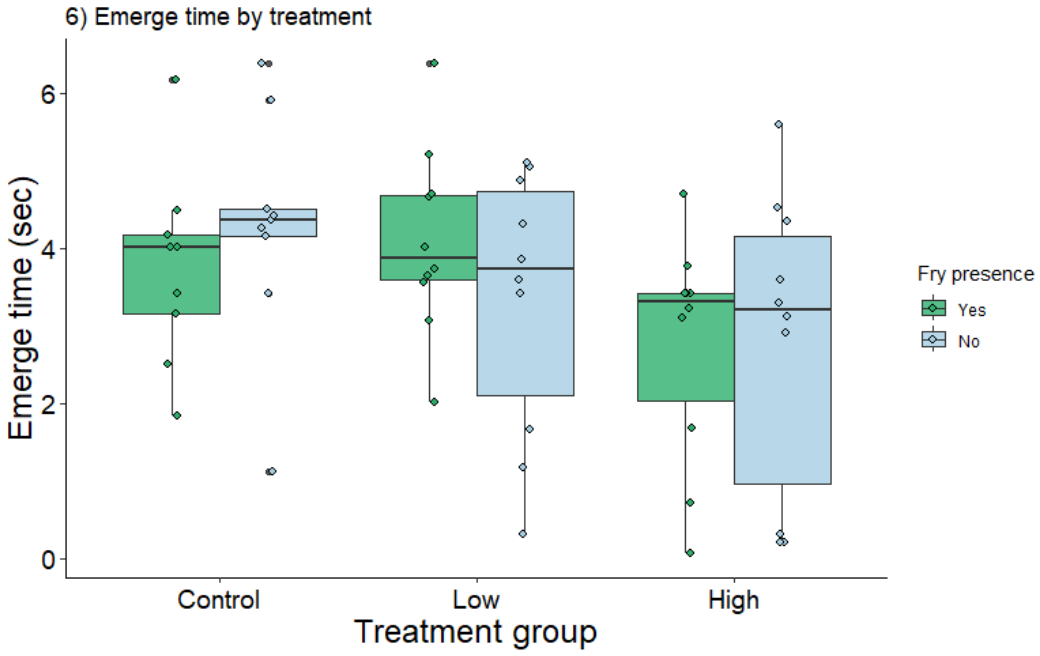

Figure 6. Log-transformed emerge time by treatment (control, low, and high) and fry presence (yes = green; no = blue). Boxes represent interquartile ranges, with horizontal lines indicating medians and individual data points jittered for clarity.

3.4 Time in front of mirror

I found no significant effects of fry presence ($P = 0.21$), or body length ($P = 0.97$) on the duration of time spent in the mirror zone ($n = 58$, Fig. 7a; Appendix C, Table C5). Post hoc comparisons showed that mirror zone duration did not differ significantly ($P > 0.45$). Females with fry in the high treatment spent the shortest time in mirror zone on average (mean = 371.38 sec, $n = 10$, SD = 150.57), while females without fry in the control treatment spent the longest (mean = 486.49 sec, $n = 8$, SD = 133.85) (Appendix C, Table C2).

I did not detect a significant effect of fry presence ($P = 0.11$), nor body length ($P = 0.24$) on the number of times that a fish visited the mirror zone ($n = 58$, Fig. 7b; Appendix C, Table C5). Post hoc comparisons showed that fish in the high treatment had significantly higher mirror zone visit rates compared to the control treatment ($P = 0.017$), while differences between the control treatment and low treatment ($P = 0.23$), and between low and high treatment ($P = 0.47$), were not significant. Although the effect of the low treatment was not statistically significant, it showed a weak visual trend (Fig. 7). On average females with fry in the high treatment made the most visits to the mirror zone (mean = 18.80 sec, $n = 10$, SD = 9.33), while females without fry in the control treatment made the fewest (mean = 4.63 sec, $n = 8$, SD = 3.66) (Appendix C, Table C2).

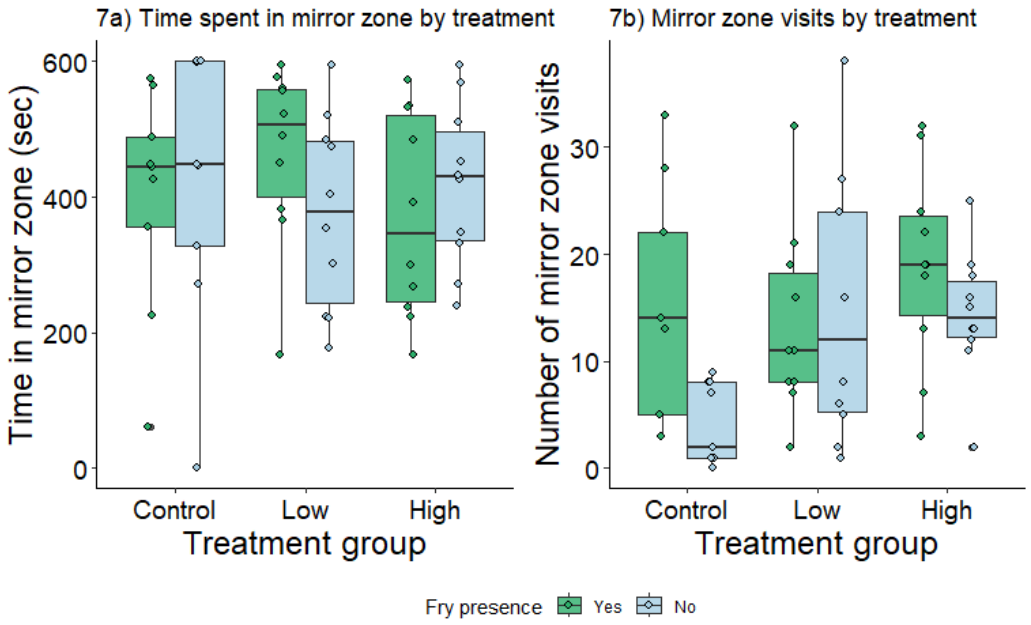

Figure 7. Mirror zone visits and mirror zone duration by treatment (control, low, and high) and fry presence (yes = green; no = blue). Boxes represent interquartile ranges, with horizontal lines indicating medians and individual data points jittered for clarity. a) Time spent in mirror zone (sec) b) Number of times a fish visited mirror zone.

4. Discussion

Research is emerging indicating that pharmaceuticals can alter fish behaviour, yet their effects on social and reproductive behaviours are still poorly understood. In this experiment, I investigated how diazepam exposure affects female reproduction and parental care, measured via mirror trials, in *Neolamprologus multifasciatus*. I found that diazepam exposure significantly reduced display aggression, while overt aggression remained unaffected, and altered reproductive output over time.

4.1 Reproductive outcomes

In this experiment, I predicted that exposure to diazepam would allow more females to reproduce by reducing stress or competition in their social environment. While the total number of reproducing females and the timing of first fry did not differ significantly between treatments, I observed a significantly steeper increase in fry numbers over time in the high treatment compared to the low treatment. This suggests that diazepam exposure at higher concentrations (37.68 ± 1.78 µg/L) increased reproductive output of *N. multifasciatus.* It is important to note that this increase was only in contrast to the low treatment, while the control treatment appeared intermediate between the low and high treatment.

One possibility is that diazepam could allow individuals to allocate energy toward reproduction, females may require less energy for territorial defence or social conflict when submitted to diazepam (Bose et al. in prep.), which reduces stress and aggression (Brodin et al. 2014; McCallum et al. 2021). Another possibility is that fry in the high treatment exhibited higher levels of activity, similar to the increased activity observed in females, which may have made them easier to detect and count. Such increased activity has been observed in fish exposed to anxiolytics, reinforcing this interpretation (Brodin et al. 2014). If fry in the control and low treatments were less active or spent more time hidden within shells, this could have led to underestimation of fry numbers in those groups. Fry was first observed in high treatment in this experiment. Although not statistically significant, it may further support the interpretation that fry exposed to higher concentrations of diazepam could become more active. This increased activity likely led them to swim outside their shells, making them more visible during observations.

Lorenzi et al. (2014) found that fathead minnows (*Pimephales promelas*) produced significantly larger clutches at low diazepam concentrations (1.04 ±

0.15 µg/L) compared to higher concentrations (13.36 ± 13.36 µg/L). In our experiment, I could not assess clutch size since females lay their eggs inside their shells, making them difficult to observe. I found no significant differences in the timing of fry appearance between treatments, which align with what Lorenzi et al. (2014) observed in their study. To better understand the long-term consequences of pharmaceutical exposure, future studies should focus on offspring growth and survival to independence.

4.2 Maternal aggression

Maternal aggression while defending offspring is an important element of parental care in *N. multifasciatus*, where females are the primary direct caregivers (Schradin & Lamprecht 2002). In this experiment, I predicted that aggression would increase with fry presence and decrease under diazepam exposure. Our findings partially support this: display aggression was significantly lower in the high treatment than the low treatment, while overt aggression remained low and was not different among the treatments. However, display aggression did not differ significantly between control and either of the treatment groups. Specifically, females in the low treatment group displayed for significantly longer durations compared to those in high treatment, suggesting that diazepam may selectively dampen non-contact, signalling behaviours more than physical attacks. Dampening of aggression aligns with previous work showing that anxiolytic compounds, like fluoxetine and oxazepam can dampen aggression in fish (Brodin et al. 2013; McCallum et al. 2017).

Interestingly, I observed a weak, but non-significant trend suggesting that females with visible fry displayed longer durations of display aggression than those without. This trend was consistent across treatments and may reflect increased parental motivation to defend offspring. In contrast, overt aggression did not differ significantly between treatments or fry presence and remained consistently low. Aggression likely reflects both offspring protection and competition over shells, a key resource for reproduction (Bose et al. 2022; Schradin & Lamprecht 2002; Zimmermann et al. 2021). Thus, diazepam may affect aggression by reducing motivation to defend offspring, shells, or territories.

A key limitation when interpreting maternal aggression in relation to fry presence is the potential misclassification of reproductive status. Some females classified as having no visible fry during filming were later observed with offspring. In the control treatment, 78% of females filmed without fry later produced fry, with an average delay of 11.0 ± 7.3 days. In the low and high treatments, this proportion was 40%, with average delays of 17 ± 16.5 and 7.5 ± 9.0 days, respectively. These findings suggest that some females may have had eggs or early-stage fry hidden

within the shell at the time of observation, which were not yet visible. Further research is required to investigate whether maternal aggression in *N. multifasciatus* varies with the developmental stage of offspring.

4.3 Time to emerge and time in front of mirror

Females in high treatment emerged from their shells significantly faster and visited the mirror zone more frequently than females in the control, suggesting increased activity. However, the duration spent in the mirror zone did not differ between treatments, suggesting that increased visits did not reflect prolonged engagement, but rather increased activity. This pattern aligns with previous research showing that exposure to anxiolytic pharmaceuticals, can elevate activity and exploratory behaviours in fish (Brodin et al. 2014; Brand et al. 2025; McCallum 2021). These changes may be attributed to diazepam's effect on the GABAA receptor, which reduces neural excitability and suppresses anxiety responses (McCallum et al. 2021). By lowering behavioural inhibition, diazepam likely encourages more exploratory movements, thus making fish more active.

4.4 Conclusion

This experiment examined how diazepam exposure affects parental aggression and reproductive output in the shell-dwelling cichlid *Neolamprologus multifasciatus*. Parental care is a key fitness-related trait, especially in species where offspring survival depends on active defence and maintenance behaviours. I hypothesised that exposure to an anxiolytic compound would dampen aggression and alter reproduction. Indeed, I found that diazepam significantly reduced display aggression and suggest that it increased exploratory behaviours and activity, while overt aggression remained unaffected. Although the number of reproducing females and timing of reproduction did not differ across treatments, fry numbers increased more rapidly in the high treatment, suggesting a treatment-related shift in reproductive output. The effects observed here primarily occurred in the high dose, which is above the concentration of diazepam measured in the environment.

These findings highlight the importance of assessing behavioural traits, particularly those linked to parental care, when evaluating the broad spectrum of effects by pharmaceutical pollutants. Because parental behaviours directly shape reproductive output and fitness, even small behavioural changes may have long-term consequences on population growth. It is therefore valuable for future ecotoxicological studies to examine traits that directly influence reproductive output, especially under chronic or multi-generational exposure scenarios.

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Popular science summary

Medications we use for ourselves and/or our animals ends up in our waterways – anything from painkillers to anti-anxiety drugs. One such medication is diazepam, a common anti-anxiety drug better known by the trade name Valium^{TM}. This type of drug, benzodiazepines, has been found in over 80 % of Europe's largest river systems. So, what happens when fish are exposed to this drug in their environment?

In this experiment, I explored how diazepam exposure affects the behaviour and reproduction of a social fish from Lake Tanganyika called *Neolamprologus multifasciatus*. These shell-dwelling cichlids live in social groups where females care for and raise their young within snail shells. A key part of parenting in this fish is to defend their offspring and nests from potential threats.

I exposed groups of these fish to different concentrations of diazepam and observed females parenting behaviour – especially aggression. To simulate a threat, I placed mirrors in their tanks close to their shells. The fish perceived their own reflections as intruders.

I found that overt aggression (attacks with physical contact) did not change, but that the duration of display aggression (warning postures) was reduced at high doses of diazepam. Meaning that the drugs dampened aggressive behaviours, which could have an impact on their ability to defend their young.

I also noticed that exposed fish emerged faster from hiding and visited the mirror more often, suggesting increased exploratory behaviours and activity. Interestingly, reproductive timing and the number of females with fry were not significantly affected by the drug. However, I did find that the number of fry produced over time was higher in high compared to low doses.

These results show that pharmaceuticals can alter important social behaviours and reproduction in fish. Since parental care and offspring defence are key to reproducing successfully, changes like this could be detrimental. Our experiment highlights the importance of considering behavioural effects when assessing the risks of pharmaceutical pollution in aquatic environments.

Appendix A

Table A1. Water quality mean and standard error per treatment (day 1 – 96).

	Control	**Low**	**High**
O2	9.82 ± 0.27	9.77 ± 0.22	9.80 ± 0.24
Nitrate	15.00 ± 6.20	14.24 ± 5.91	13.23 ± 4.51
Nitrite	0 ± 0	0 ± 0	0 ± 0
GH	8.18 ± 1.49	7.49 ± 1.30	7.19 ± 1.041
KH	6.00 ± 0.47	6.087 ± 0.42	5.96 ± 0.20
pH	7.91 ± 0.56	8.075 ± 0.38	8.13 ± 0.38
Cl	0 ± 0	0 ± 0	0 ± 0
Temp	26.48 ± 0.81	27.095 ± 0.68	26.93 ± 0.51

Table A2. Summary of model selection and interaction statistics for behavioural trials (AIC, df, LRT, and P).

Behaviour	**df**	**AIC**	**LRT**	***P***
Display aggression				
Treatment x fry	2	739.67	1.38	0.50
Body length	1	741.14	0.85	0.36
Overt aggression				
Treatment x fry	2	209.58	0.011	0.99
Body length	1	212.01	0.44	0.51
Mirror zone counts				
Treatment x fry	2	417.89	4.87	0.089
Body length	1	415.32	0.3	0.58
Mirror zone duration				
Treatment x fry	2	734.74	1.49	0.48
Body length	1	736.78	1.53	0.22
Latency to emerge				
Treatment x fry	2	223.81	1.81	0.40
Body length	1	223.81	0.0039	0.95

Table A3. Summary of model selection and interaction statistics for reproductive output (AIC, df, LRT, and P).

Behaviour	**df**	**AIC**	**LRT**	***P***
Females with fry				
Treatment x day	2	689.30	1.11	0.57
Amount of fry				
Treatment x day	2	1129.5	8.23	**0.016***
Timing				
Treatment x fry	2	417.89	4.87	0.089

Appendix B

*Table B1. Effects of treatment (control, low, and high) on reproductive output. Includes number of females with fry, amount of observed fry, and timing of first fry observation., with estimate ± SE, z-value, p-value). P (P < 0.05) are indicated in **bold** and marked with *****.*

	Estimate ± SE	**z value**	***P* value**
Females with fry			
Effects			
Low treatment	–0.71 ± 0.48	–1.49	0.135
High treatment	0.11 ± 0.46	0.25	0.80
Day	0.033 ± 0.0033	10.22	**< 0.0001***
Contrasts			
Control vs. Low	0.71 ± 0.48	1.49	0.29
Control vs. High	–0.11 ± 0.46	–0.25	0.97
Low vs. High	–0.83 ± 0.48	–1.73	0.19
Amount of fry			
Effect			
Low treatment	–0.34 ± 0.86	–0.40	0.69
High treatment	–0.61 ± 0.82	–0.75	0.46
Day	0.036 ± 0.0042	8.59	**< 0.0001***
Low treatment – day	–0.0086 ± 0.0066	–1.31	0.19
High treatment – day	0.010 ± 0.0060	1.69	0.091
Contrasts			
Control vs. Low	0.0086 ± 0.0066	1.31	0.39
Control vs. High	–0.010 ± 0.0060	–1.69	0.21
Low vs. High	–0.019 ± 0.0066	–2.82	**0.013***
Timing of reproduction			
Effects			
Low treatment	6.26 ± 6.62	0.95	0.35
High treatment	5.89 ± 6.24	0.94	0.35
Contrasts			
Control vs. Low	–6.26 ± 6.62	–0.95	0.62
Control vs. High	–5.89 ± 6.24	–0.94	0.62
Low vs. High	0.367 ± 6.87	0.053	1.00

Appendix C

*Table C1. Results from mixed-effects models for display and overt aggression. Effects and contrasts for behavioural measures, with estimates ± standard error, z values and P (P < 0.05) are indicated in **bold** and marked with *****.*

	Estimate ± SE	z value	*P*
Display aggression			
Effect			
Low treatment	41.34 ± 37.86	1.092	0.27
High treatment	–54.27 ± 37.83	–1.44	0.15
Fry (No)	–55.09 ± 30.79	–1.79	0.074
Body length	–97.30 ± 87.98	–1.11	0.27
Contrast			
Control vs. Low	–41.3 ± 37.9	–1.092	0.52
Control vs. High	54.3 ± 37.8	1.44	0.33
Low vs. High	95.6 ± 36.9	2.59	**0.033 ***
Overt aggression			
Effect			
Low treatment	0.34 ± 0.48	0.71	0.48
High treatment	–0.16 ± 0.54	–0.30	0.77
Fry (No)	–0.45 ± 0.42	–1.092	0.28
Body length	0.75 ± 1.11	0.68	0.50
Contrasts			
Control vs. Low	–0.34 ± 0.48	–0.71	0.76
Control vs. High	0.16 ± 0.54	0.30	0.95
Low vs. High	0.50 ± 0.47	1.058	0.54

Table C2. Mean duration for display aggression, mean counts for overt aggression, as well as mean counts and duration for mirror visits and emerge time across control, low and high treatment groups, with mean and standard deviation.

Treatment	Fry	Mean	SD	Number
Display aggression				
Control	Yes	248.52	150.49	8
	No	151.82	133.42	8
Low	Yes	245.75	113.48	10
	No	188.57	133.025	10
High	Yes	143.36	69.64	10
	No	118.032	115.44	9
Overt aggression				
Control	Yes	5.50	5.066	4
	No	3.33	3.21	3
Low	Yes	4.00	2.00	6

	No	6.50	4.20	4
High	Yes	2.00	1.00	5
	No	4.67	4.041	3
Mirror zone counts				
Control	Yes	15.00	10.82	9
	No	4.63	3.66	8
Low	Yes	13.50	8.71	10
	No	15.10	12.61	10
High	Yes	18.80	9.33	10
	No	14.40	6.00	10
Mirror zone duration				
Control	Yes	398.70	164.90	9
	No	486.49	133.85	8
Low	Yes	466.81	131.65	10
	No	375.68	142.74	10
High	Yes	371.38	150.57	10
	No	417.63	120.14	10
Emerge time				
Control	Yes	90.23	147.22	9
	No	152.57	194.13	9
Low	Yes	118.17	172.68	10
	No	64.97	63.54	10
High	Yes	29.95	81.32	10
	No	54.43	31.14	10

Table C4. Number of females (with and without fry) and average duration spent in the shell (sec) across control, low, and high treatment groups, with mean ± standard deviation.

Treatment	Fry	Mean ± SD	Number
Control	Yes	10.88 ± 8.10	4
	No	49.89 ± 40.78	5
Low	Yes	30.38 ± 30.34	8
	No	54.60 ± 58.79	5
High	Yes	45.18 ± 42.93	7
	No	50.35 ± 37.82	7

*Table C5. Results from mixed-effects model for mirror zone interactions and latency to emerge, with estimate ± standard error, z-value, and P ($P < 0.05$) are indicated in **bold** and marked with *.*

	Estimate ± SE	z value	*P*
Mirror zone counts			
Effect			

Low treatment	0.40 ± 0.25	1.64	0.10
High treatment	0.65 ± 0.23	2.75	**0.0061 ***
Fry (No)	–0.29 ± 0.18	–1.61	0.11
Body length	0.59 ± 0.50	1.18	0.24
Contrasts			
Control vs. Low	–0.40 ± 0.25	–1.64	0.23
Control vs. High	–0.65 ± 0.24	–2.75	**0.017 ***
Low vs. High	–0.24 ± 0.21	–1.18	0.47
Mirror zone duration			
Effects			
Low treatment	18.50 ± 40.87	0.45	0.65
High treatment	–29.97 ± 40.83	–0.73	0.46
Fry (No)	41.36 ± 33.23	1.24	0.21
Body length	3.39 ± 94.96	0.036	0.97
Contrasts			
Control vs. Low	–18.5 ± 40.9	–0.45	0.89
Control vs. High	30.0 ± 40.8	0.73	0.74
Low vs. High	48.5 ± 39.9	1.22	0.45
Latency to emerge			
Effects			
Low treatment	0.35 ± 0.67	0.52	0.60
High treatment	–1.00 ± 0.67	–1.50	0.13
Fry (No)	0.53 ± 0.69	0.77	0.44
Body length	0.072 ± 1.16	0.062	0.95
Low treatment – fry no	–1.30 ± 0.97	–1.34	0.18
High treatment – fry no	–0.47	0.95	0.62
Contrasts			
Control vs. Low	0.30 ± 0.47	0.63	0.80
Control vs. High	1.23 ± 0.47	2.61	**0.032 ***
Low vs. High	0.93 ± 0.46	2.025	0.12

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