UMEÅ UNIVERSITET

Pharmaceutical drugs in aquatic ecosystems

Does exposure to oxazepam alter behavior of brown trout (*Salmo trutta*) and consequently affect the dominance hierarchy?

Olivia Jirlén

Examensarbete, 15 hp
Kandidatprogrammet i biologi och geovetenskap med inriktning biologi, 180 hp
Vt 2022

Abstract

A greater consumption of pharmaceutical drugs entails an increased concentration of active benzodiazepines in aquatic ecosystems. Consequently, aquatic organisms are experiencing altered behavior that may affect dominance hierarchies since social status (among other variables) is associated with behavioral traits. The aim of this study was to determine whether dominance hierarchies of brown trout (*Salmo trutta*) were affected by exposure to a benzodiazepine (oxazepam). Hypothetically, aggression (in dominants) and anxiety (in subordinates) will reduce following exposure. The fish should consequently display a significant disparity between treatment groups regarding the frequency of dominance change (i.e., who is dominant versus subordinate). This research included behavioral coding of 150 juvenile brown trout (*Salmo trutta*) divided into 50 size-matched social groups of 3 individuals. Each group was exposed to one of three oxazepam concentrations (30 µg/L, 1.5 µg/L and 0 µg/L). The results indicate no relationship between an altered aggression and oxazepam exposure. In addition, the level of aggression reduced over time (regardless of social status and concentration) and the initial subordinates remained significantly less aggressive than the initial dominants. The frequency of dominance change did not differ significantly between different treatment groups. Body size did not affect social status. The results in low treatment groups may be due to a low bioconcentration since previous research exhibited similar results. However, the lack of results in high treatment groups could be due to something else. In conclusion, the dominance hierarchy was not disrupted by oxazepam exposure because aggression was unaffected.

Key words
Dominance hierarchy, behavior and oxazepam

Table of contents

1. Introduction

1.1 Background

The consumption of pharmaceutical drugs has increased during the last decades and is now recognized as a major threat to aquatic ecosystems (Bernhardt, Rosi and Gessner 2017; Brodin et al. 2013). Psychiatric pharmaceuticals such as anxiolytics and antidepressants (for example benzodiazepines) are commonly used to treat anxiety and clinical depression (Petty et al. 1995). However, several benzodiazepines enter aquatic ecosystems via treated wastewater and due to a resistance among benzodiazepines to photodegradation (partially because of humic acids), these anxiolytics remains biochemically active (Brodin et al. 2017; Calisto, Domingues and Esteves 2011). Sediment cores collected in 1995 and 2013 indicate that anxiolytics have persisted in the sediments since they were deposited in the early 1970s despite degradation processes and sediment diagenesis (Klaminder et al. 2015).

Benzodiazepines have a pharmacological effect by binding to type A of gamma-aminobutyric acid ($GABA_A$) receptors and consequently hyperpolarize the neuron by forming an anion channel (Korpi, Gründer and Lüddens 2002; Khan 2021). Hence, benzodiazepines inhibit nerve signals, which can alter behavioral traits (Khan 2021; Brodin et al. 2013). Orthologs (i.e., shared genes among species) for human drug targets relevant for ecotoxicity have been detected in several aquatic animal species as well as green algae (Gunnarsson et al. 2008; Brodin et al. 2013). As a consequence, aquatic organisms may experience similar therapeutic effects of anxiolytics as humans (Brodin et al. 2013).

Oxazepam (a benzodiazepine) is the most prescribed anxiolytic in Sweden and was in 2013 found in Swedish aquatic ecosystems at concentrations ranging from 2.9 $ng*L^{-1}$ to 12.4 $ng*L^{-1}$ with no seasonal variation (Klaminder et al. 2015; Wang et al. 2017). Due to similar concentrations of oxazepam, wild roach (*Rutilus rutilus*) have experienced an enhanced boldness and level of activity (Brodin et al. 2017). In the presence of predators, such behavioral alterations may increase the mortality rate and consequently reduce the population size of the affected species (Brodin et al. 2017). For instance, a dilute concentration of oxazepam (encountered in surface waters near wastewater effluent) affected migration dynamics of Atlantic salmon (*Salmo salar*) smolt in terms of their migration intensity (Hellström et al. 2017). Consequently, survival and fitness of salmon smolt may decline and hence, a disruption in salmon migration may result in severe ecological consequences (Hellström et al. 2017).

A variety of animal species (including fish) live in groups that are organized into dominance hierarchies where social status determines access to key resources (McCallum et al. 2021). Since dominant individuals commonly occupy the most profitable positions in a hierarchy, dominant males often acquire a higher reproductive success in contrast to subordinate males (Sloman and Armstrong 2005; Ellis 1995). As social status is associated with behavioral traits such as aggression and stress dynamics, exposure to benzodiazepines in the natural environment may affect animal dominance hierarchies (McCallum et al. 2021; Creel 2021).

1.2 Aim and research questions

The aim of this study is to assess whether the dominance hierarchy of brown trout (*Salmo trutta*) was affected by exposure to oxazepam (of several concentrations). This was determined by whether the level of aggressive acts per day was different between the social statuses (i.e., the initial dominants versus the initial subordinates) and if it differed between

different treatment groups of the same social status. Moreover, the frequency of dominance change (i.e., a switch in social status) in different treatment groups was analyzed.

1.3 Hypothesis

Oxazepam is designed to inhibit nerve signals (Khan 2021), which theoretically will reduce aggression (in dominants) and anxiety (in subordinates). Therefore, the level of aggressive acts have the potential to be more equal between the social statuses since initial subordinates may be more willing to fight for the key resources (due to less anxiousness). As a consequence, the level of aggressive acts may not decrease over time in exposed groups due to ongoing competitions. This suggests that exposure to oxazepam may result in a higher frequency of dominance change (i.e., a switch in social statuses), altogether disrupting the dominance hierarchy.

2. Method and material

This research was conducted under a Jordbruksverket ethical permit to Tomas Brodin (number Dnr 5.9.18-17028/2020).

2.1 Collection and housing

Initially, 150 juvenile brown trout (*Salmo trutta*) were sourced from the Norrfors Fish Hatchery (close to Umeå, Sweden) and the water in that area (from Ume river) receives no known source of pharmaceutical pollution (McCallum et al. 2021). The fish were transported to Umeå University and were housed for 72 h in 1000 L flow-through tanks filled with ground water to facilitate recovery. Each tank contained about 50 individuals in order to prevent dominance hierarchies from forming in the housing tanks. In addition, the fish were fed food pellets (INICIO, BioMar, Denmark) until satiation once per day.

2.2 Formation of social groups

The experiment included 5 replicates with 10 social groups per replicate. Each social group contained 3 size-matched individuals according to total length (cm) and body mass (g) (accuracy of 0.01 g), which were measured parallel to standard length (cm). In order to reduce any stress during size-matching, the fish were anesthetized using MS-222 (Ethyl 3-aminobenzoate methanesulfonate, CAS number: 886-86-2) with a concentration of 0.1 g/L. In addition, fin damage (dorsal, caudal and pectoral) was scored for each fish according to a 5-level damage scale established by Hoyle et al (2007, 142-148) (data not included in this research) and lastly assigned a unique dorsal fin clip in order to facilitate identification during behavioral trials. Each social group were thereafter returned to a dark, aerated tank in order to recover from sedation before transferred into a 112 L glass aquarium filled with ground water in a climate-controlled room. The glass aquarium contained a re-circulating water pump, an air stone and a plastic plant. The plastic plant simulated a desirable resource and would therefore likely be a profitable position to be occupied by the dominant individual. The tanks were left to acclimate (except for daily feeding) for 36 h, which allowed dominance hierarchies to form before exposure to oxazepam.

2.3 Exposure and behavioral coding

Each social group was recorded 4 times (including one pre-exposure trial) between 9.00 and 10.00 am (60 min) over a period of 7 days using camcorders (2.64 MP/1080p, Sony HDR-PJ50) mounted on tripods. All equipment as well as experimenters were concealed behind opaque blinds in order to reduce disturbing the fish. Following the pre-exposure trial, 3 separate concentrations (30 µg/L, 1.5 µg/L and 0 µg/L) of oxazepam were randomly distributed within each replicate and added by pipetting the amount of stock solution required in order to obtain the desired concentration. The stock solution was prepared by dissolving oxazepam (Merck; CAS number: 604-75-1) in water purified by a Milli-Q Gradient water system (Millipore, Billerica, Massachusetts, USA). The selected concentrations would represent a pharmaceutically polluted habitat (1.5 µg/L) and human therapeutic levels (30 µg/L). Following the final post-exposure trial, all fish were euthanized by an overdose (0.3 g/L) of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, CAS number: 886-86-2) in order to re-measure body mass (g) and collect plasma as well as scoring fin damage (dorsal, caudal and pectoral) (data not included in this research). All fish and plasma samples were stored in -20^{o}C for further chemical analyses (data not included in this research) regarding their level of oxazepam.

Each trial was coded (blind to concentration of oxazepam) according to aggressive interactions following an ethogram (appendix 5). An aggressive interaction included one aggressor and one receiver. As the total number of aggressions given (per individual) was subtracted by the total number of aggressions received (per individual), each individual was consequently assigned a social status as dominant (1 per social group) or subordinate (2 per social group). In addition, an individual's occupation of the plastic plant was recorded as a further act of dominance. All behavioral coding was conducted using the open-source software BORIS.

2.3.1 Determination of social status

Determination of an individual´s social status was determined by the relationship between their aggressive acts and their occupation of the plastic plant (figure 1). However, as discrepancies (no aggression or monopolization) occurred, one variable was given superiority regarding social status. Since monopolization of the plant resource is due to a resolved competition (Sloman and Armstrong 2005), aggression without monopolization suggests an ongoing competition. As a consequence, an individual's occupation of the plastic plant resource indicated their social status versus relying on aggression.

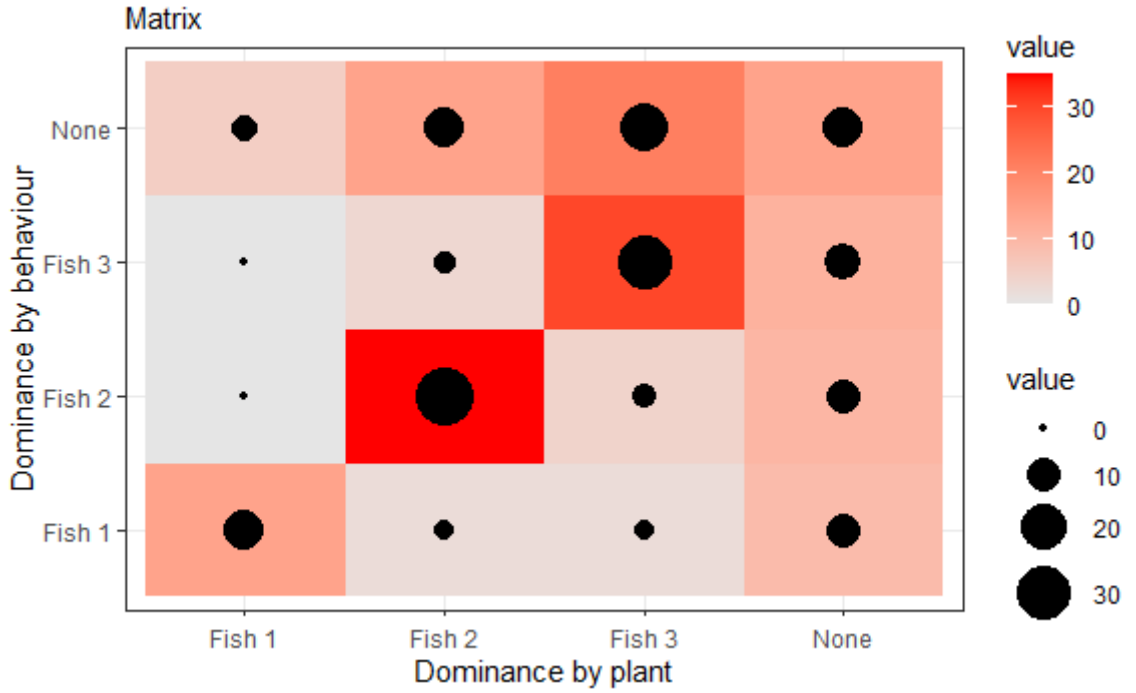

Figure 1. A summation (of each trial) of dominance per fish according to aggressive behavior and occupation of the plastic plant. Aggressive behavior is shown by color (deeper color indicates more aggressive acts) and monopolization is shown by circles (a larger circle indicates more frequent monopolization). Trials with no dominant individual were scored as "none".

2.4 Statistical analyses

All statistical analyses were conducted using the open-source software R (version 4.1.3).

2.4.1 Aggressive acts per day (in accordance with status and concentration)

With aggressive behavior as a dependent variable, a linear regression analysis was used in order to examine if aggression changed over time or in relation to social status or oxazepam exposure (i.e., concentration of oxazepam). Whether the data was normally distributed and

homoscedastic was visually examined through a residuals-versus-fitted plot as well as a Shapiro-Wilk test. The variable was then log-transformed in order to meet these assumptions.

2.4.2 Dominance change

Since social status is a categorical variable, a Chi-square test was used in order to examine if social status changed with a higher frequency in exposed versus unexposed social groups.

2.4.3 Social status relative to body size

A generalized linear model was conducted in order to analyze the significance of a continuous variable (body mass (g) and body length (cm) separately) on a binary response (social status).

3. Results

3.1 Aggression per day in accordance with status and concentration

The level of aggressive behavior decreased with time regardless of social status and concentration of oxazepam (estimate ± SE = -0.037 ± 0.0088 , t = -4.21, $p < 0.0001$) (appendix 2; figure 2). However, the initial subordinate individuals remained significantly less aggressive compared to the initial dominant individuals (estimate ± SE = -0.55 ± 0.036, t = -15.32, $p < 0.0001$) (appendix 2; figure 2). Despite a visible discrepancy (figure 2), there was no significant correlation between an altered aggression and exposure to oxazepam (1.5 µg/L to control: estimate ± SE = -0.0083 ± 0.42, t = -0.20, p = 0.84; 30 µg/L to control: estimate ± SE = -0.014 ± 0.041, t = -0.35, p = 0.73) (appendix 2).

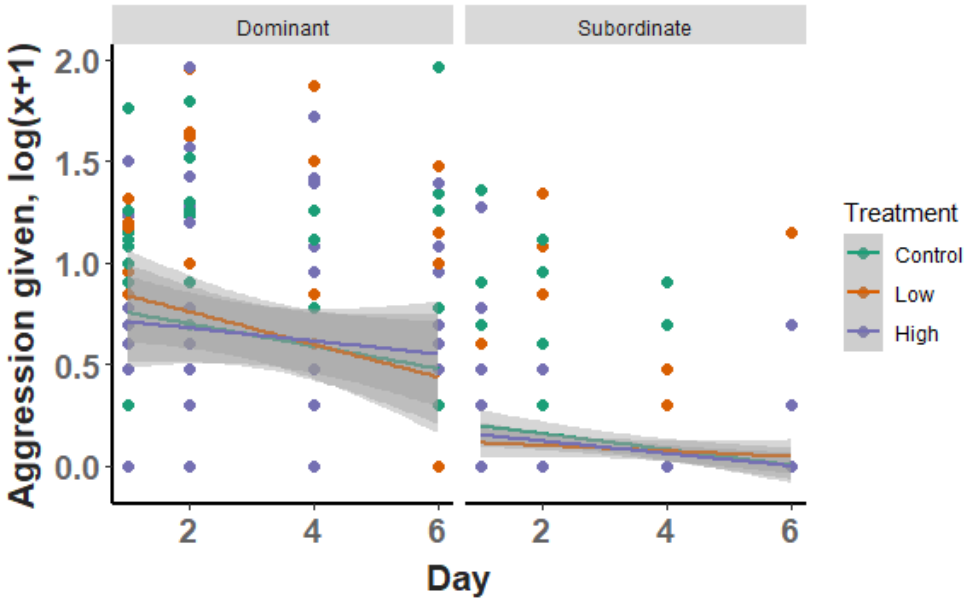

Figure 2. Aggression given (y-axis) per day (x-axis) in accordance with social status (dominant or subordinate) and treatment group (30 µg/L, 1.5 µg/L or 0 µg/L of oxazepam). The values of aggression given has been log-transformed in order to express a normal distribution. Values associated with control (0 µg/L) trials are green whereas low concentration (1.5 µg/L) trials are red and high concentration (30 µg/L) trials are purple. Each treatment group (per social status) express a linear gradient based on their respective values (represented by their respective color).

3.2 Dominance change

The amount of trials containing a dominance change among control groups was approximately 25 % (figure 3). A 3-sample test indicated a non-significance regarding the

probability of a difference in dominance change between concentrations of oxazepam (X^2 = 0.169, df = 2, p = 0.919) (appendix 3). In addition, a 2-sample test between trials of control groups and trials of exposed groups (1.5 µg/L and 30 µg/L analyzed together) also displayed non-significance ($X^2 = 1.064 * 10^{-30}$, df = 1, p = 1 (appendix 3).

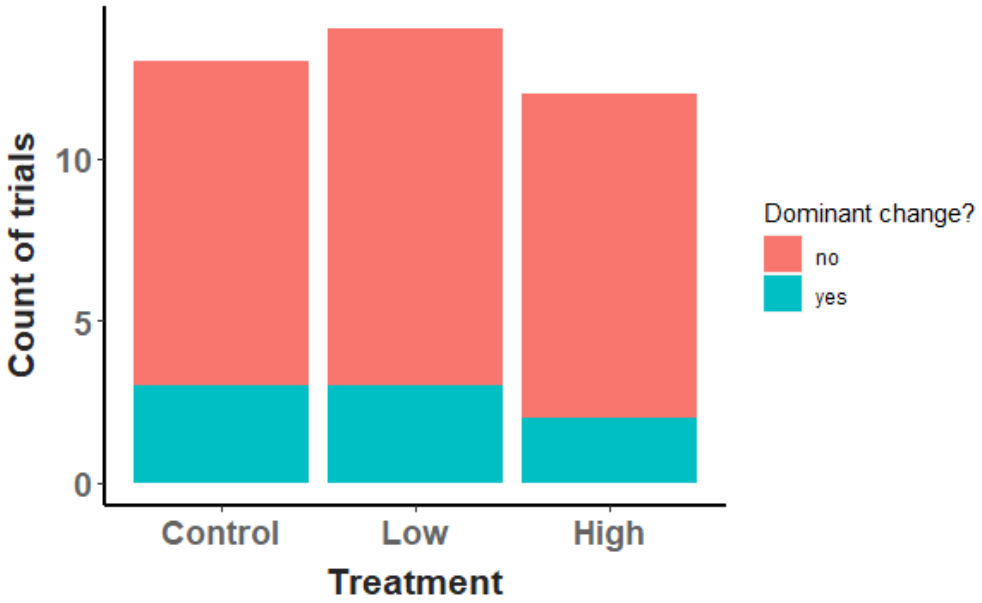

Figure 3. Number of trials containing a dominance change (indicated by blue), implying a replacement of the initial dominant individual by a new individual. The number of trials with no dominance change is shown in red. Moreover, as a result of trials containing no dominant individual, the y-axis (count of trials) differ between concentrations.

3.3 Social status relative to body size

Average body mass (g) and body length (cm) did not differ significantly between dominants and subordinates (figure 4; body mass (g): estimate ± SE = -0.001 ± 0.008, z = -0.14, p = 0.886; body length (cm): estimate ± SE = -0.03 ± 0.083, z = -0.356, p = 0.721) (appendix 4). Consequently, body size had a non-significant effect on social status.

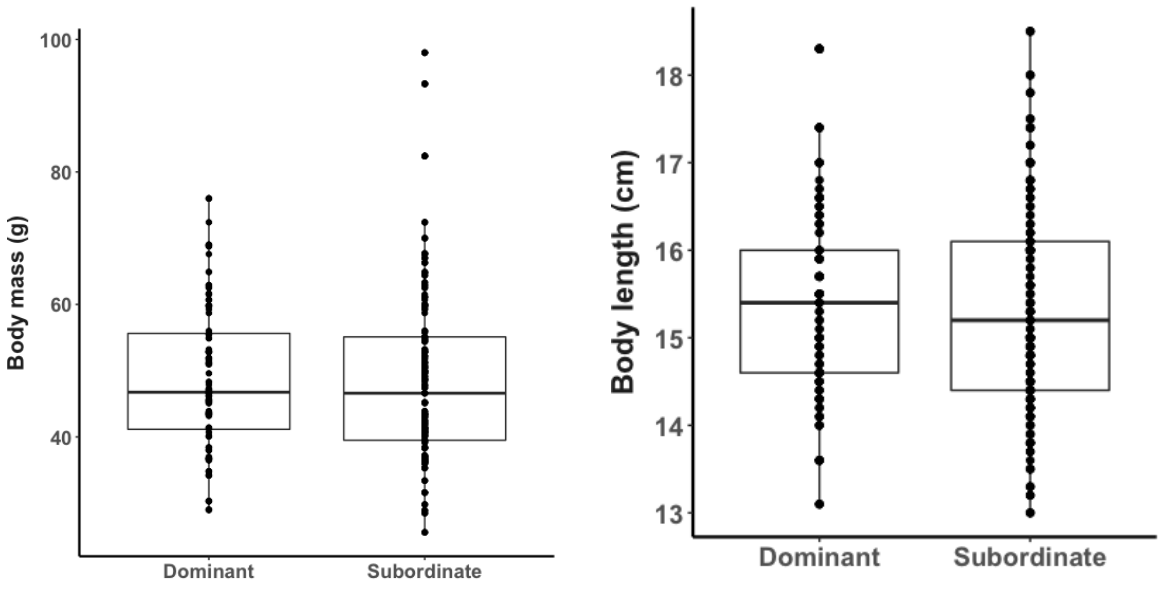

Figure 4. Average body mass (g) (left figure) and average body length (cm) (right figure) as contineous variables (y-axis) in relation to a binary response (dominant or subordinate). The results determine whether the dominant fish is the larger fish relative to body size.

4. Discussion

Dominance hierarchies are common among juvenile salmonid species (including *Salmo trutta*) as a consequence of intra-species competition and are associated with behavioral aggression and anxiety (Sloman 2011; McCallum et al. 2021). Hence, a modification of such behavioral traits (due to oxazepam exposure) may hypothetically equalize the hierarchy by lessening aggression in dominants and anxiety in subordinates. Consequently, exposed groups would be predicted to display a higher frequency of dominance change due to an equal level of aggression between social statuses. However, the current study shows no significant relationship between an altered aggression and exposure to oxazepam. The level of aggression decreased with time regardless of treatment group and social status, with the initial subordinate individuals remaining significantly less aggressive. Consequently, the frequency of dominance change in exposed groups remained equal to control groups. Body size also had no effect on social status, indicating that the size-matching in this study was effective.

Previous research has found similar results in rainbow trout (*Oncorhynchus mykiss*) during exposure to the pharmaceutical drug citalopram (a selective serotonin reuptake inhibitor) (Holmberg et al. 2011). The result was a consequence of a relatively low bioconcentration, which further research connected to a low exposure concentration (Holmberg et al. 2011; Huerta et al. 2016). This relationship is further exhibited in fathead minnow (*Pimephales promelas*) as no behavioral effects was found during exposure to 0.8 μg/L of oxazepam (Huerta et al. 2016). Henceforth, the non-significant effect on aggression in low treatment groups of brown trout (*Salmo trutta*) may be due to a low bioconcentration (of oxazepam). However, the similar results in high treatment groups may be due to something else since previous research has connected a high exposure to high tissue bioconcentrations (Huerta et al. 2016). Fathead minnow (*Pimephales promelas*) experience similar results as brown trout (*Salmo trutta*) during high exposure treatments and further research was suggested to examine the cause of this result (Huerta et al. 2016).

On the other hand, several fish species (European perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*)) have shown an increase in boldness and activity among subordinate individuals during oxazepam exposure (Brodin et al. 2013; Brodin et al. 2017). Such behavioral alterations caused a higher foraging success due to an increased feeding rate (McCallum et al. 2021; Brodin et al. 2013). Henceforth, a high growth rate will be achievable without an increase in aggression and under those circumstances having less effects on aggression. This could explain the non-significant effect on aggression in brown trout (*Salmo trutta*) in this study (regardless of treatment group). Yet, an increased feeding rate in the wild will probably entail a higher predation risk which may decrease the effective population (i.e., the number of reproducing individuals). Moreover, a higher growth rate in subordinates should decrease the bimodal size distribution during reproduction, hence having a negative effect on alternative reproductive tactics (ARTs). This is due to an earlier smoltification (approximately one year earlier) among dominant salmonids and by so contribute to ARTs (Sloman 2011). Regardless, the dominance hierarchy may not be disrupted since aggression remains unaffected in this study.

Previous research shows a bioaccumulation of oxazepam in brown trout (*Salmo trutta*) and European perch (*Perca fluviatilis*) (McCallum et al. 2021; Brodin et al. 2013). This may affect fish-consuming terrestrial species just as bioaccumulation of PCB and DDT (fat-soluble environmental toxins) in fish affected common murres (*Uria aalge*) (Previšić et al. 2021;

Holm 2022; Miljökonsultgruppen u.å). Henceforth, people in no need of anxiolytics could be exposed via fish consuming and potentially experience the therapeutic effects.

4.1 Conclusion

In conclusion, exposure to oxazepam has no effect on aggression (regardless exposure concentration) in brown trout (*Salmo trutta*). The result in low treatment groups may be due to a low bioconcentration of oxazepam since previous research exhibit similar results in other fish species. However, the result in high treatment groups should be due to something else. The result indicates that the dominance hierarchy may not be disrupted since aggression remains unaffected.

4.2 Sources of error

Fish mortality occurred 2 times in 1 replicate (tank 4 and 5 in replicate 2) during behavioral trials, which may have affected the results. However, 96 % (48/50) of all trials contained a social group of 3 individuals, which hence suggests a non-significant error. Furthermore, as several coders contributed to behavioral coding, a significant disparity may have occurred regarding the number of aggressive acts per individual. Several trials were coded cooperatively among each coder, which hypothetically would have reduced a possible disparity in coding skills. However, a statistical analyze of inter-rater reliability would have facilitated for further support.

5. Acknowledgements

The author would like to thank E. McCallum (supervisor) and A. Bose for support during behavioral coding and subsequent statistical analyzes. In addition, the author would like to thank her collaborator during behavioral coding.

6. References

Bernhardt, E., Rosi, E. and Gessner, M. 2017. Synthetic chemicals as agents of global change. *Frontiers in Ecology and the Environment* 15(2): 84-90. https://doi-org.proxy.ub.umu.se/10.1002/fee.1450

Brodin, T., Fick, J., Jonsson, M. and Klaminder, J. 2013. Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science* 339(6121): 814-815. doi: 10.1126/science.1226850

Brodin, T., Nordling, J., Lagesson, A., Klaminder, J., Hellström, G., Christensen, B. and Fick, J. 2017. Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish, (*Rutilus rutilus*). *Journal of Toxicology and Environmental Health* 80(16-18): 963-970. https://doi-org.proxy.ub.umu.se/10.1080/15287394.2017.1352214

Calisto, V., Domingues, R. and Esteves, V. 2011. Photodegradation of psychiatric pharmaceuticals in aquatic environments – Kinetics and photodegradation products. *Water Research* 45(18): 6097-6106. https://doi.org/10.1016/j.watres.2011.09.008

Creel, S. 2001. Social dominance and stress hormones. *Trends in Ecology & Evolution* 16(9): 491-497. https://doi.org/10.1016/S0169-5347(01)02227-3

Ellis, L. 1995. Dominance and reproductive success among nonhuman animals: Across-species comparison. *Ethology and Sociobiology* 16(4): 257-333. https://doi.org/10.1016/0162-3095(95)00050-U

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O. and Larsson, J. 2008. Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. *Environmental Science & Technology* 42(15): 5807-5813. https://doi-org.proxy.ub.umu.se/10.1021/es8005173

Hellström, G., Klaminder, J., Finn, F., Persson, L., Alanära, A., Jonsson, M., Fick, J. and Brodin, T. 2016. GABAergic anxiolytic drug in water increases migration behaviour in salmon. *Nature Communications* 7(13460). https://doi-org.proxy.ub.umu.se/10.1038/ncomms13460

Holm. S-O. 2022. *Kustviltet – Ekologi och förvaltning*. [Powerpoint]. https://www.canvas.umu.se/courses/5687/files/folder/Kursmaterial/F%C3%B6rel%C3%A4sningar?preview=799992 (Retrieved 2022-05-25)

Holmberg, A., Fogel, J., Albertsson, E., Fick, J., Brown, J., Paxéus, N., Förlin, L., Johnsson, J. and Larsson, J. 2011. Does waterborne citalopram affect the aggressive and sexual behaviour in rainbow trout and guppy?. *Journal of Hazardous Materials* 187(1-3): 596-599. doi: https://doi.org/10.1016/j.jhazmat.2011.01.055

Hoyle, I., Oidtmann, B., Ellis, T., Turnbull, J., North, B., Nikolaidis, J. and Knowles, T. G. 2007. A validated macroscopic key to assess fin damage in farmed rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 270(1-4): 142-148. https://doi.org/10.1016/j.aquaculture.2007.03.037

Huerta, B., Margiotta-Casaluci, L., Rodríguez-Mozaz, S., Scholze, M., Winter, M., Barceló, D. and Sumpter, J. 2016. Anti-anxiety drugs and fish behavior: Establishing the link between internal concentrations of oxazepam and behavioral effects. *Environmental Toxicology and Chemistry* 35(11): 2782-2790. https://doi.org/10.1002/etc.3448

Khan, S. 2021. *HOW DO BENZODIAZEPINES WORK?*. https://www.rxlist.com/anxiolytics_benzodiazepines/drug-class.htm (Retrieved 2022-02-25)

Klaminder, J., Brodin, T., Sundelin, A., Anderson, N. J., Fahlman, J., Jonsson, M. and Fick, J. 2015. Long-Term Persistence of an Anxiolytic Drug (Oxazepam) in a Large Freshwater Lake. *Environmental Science & Technology* 49(17): 10406-10412. https://doi.org/10.1021/acs.est.5b01968

Korpi, E., Gründer, G. and Lüddens, H. 2002. Drug interactions at $GABA_A$ receptors. *Progress in Neurobiology* 67(2): 113-159. https://doi.org/10.1016/S0301-0082(02)00013-8

McCallum, E., Dey, C., Cerveny, D., Bose, A. and Brodin, T. 2021. Social status modulates the behavioral and physiological consequences of a chemical pollutant in animal groups. *Ecological Applications* 31(8). https://doi-org.proxy.ub.umu.se/10.1002/eap.2454

Miljökonsultgruppen. u.å. *Miljö- och hälsopåverkan*. http://www.sanerapcb.nu/web/page.aspx?refid=10 (Retrieved 2022-05-25)

Petty, F., Trivedi, M. H., Fulton, M. and Rush, A. J. 1995. Benzodiazepines as antidepressants: does GABA play a role in depression?. *Biological Psychiatry* 38(9): 578-591. doi: 10.1016/0006-3223(95)00049-7

Previšić, A., Vilenica, M., Vučković, N., Petrović, M. and Rožman, M. 2021. Aquatic Insects Transfer Pharmaceuticals and Endocrine Disruptors from Aquatic to Terrestrial Ecosystems. *Environmental Science & Technology* 55(6): 3736-3746. https://doi.org/10.1021/acs.est.0c07609

Sloman, K. A. and Armstrong J. D. 2005. Physiological effects of dominance hierarchies: laboratory artefacts or natural phenomena?. *Journal of Fish Biology* 61(1): 1-23. https://doi.org/10.1111/j.1095-8649.2002.tb01733.x

Sloman, K. A. 2011. SOCIAL AND REPRODUCTIVE BEHAVIORS | Dominance Behaviors. *Encyclopedia of Fish Physiology* 649-655. https://doi.org/10.1016/B978-0-12-374553-8.00082-4

Wang, C., Hou, L., Li, J., Xu, Z., Gao, T., Yang, J., Zhang, H., Li, X. and Du, P. 2017 Occurrence of diazepam and its metabolites in wastewaters and surface waters in Beijing. *Environmental Science and Pollution Research* 24: 15379-15389. doi: 10.1007/s11356-017-8922-8

Appendix

Appendix 1. The W-value and the p-value determined by a Shapiro-Wilks test for normal distribution of aggressive behaviors among all behavioral trials.

W-value	p-value
0.884	< 0.0001

Appendix 2. Significance of number of aggressive behaviors per (per treatment group) with a further examination of significance between social statuses (regardless concentration of oxazepam) and between control trials and exposed trials (1.5 µg/L to control and 30 µg/L to control). These statistical analyses were conducted via a simple linear regression analysis and presented by the estimate value, standard error (SE), t-value and p-value.

Coefficients	Estimate	SE	t-value	p-value
Dominants vs subordinates	-0.55	0.036	-15.32	<0.0001
Day	-0.038	0.0088	-4.21	<0.0001
Low treatment	-0.0083	0.042	-0.20	0.84
High treatment	-0.014	0.041	-0.35	0.73

Appendix 3. Probability of a difference in dominance change between each treatment group. The 3-sample test implies an examination of all concentrations (30 µg/L, 1.5 µg/L and 0 µg/L) whereas the 2-sample test examine the exposed trials (30 µg/L and 1.5 µg/L). These statistical analyses were conducted via a Chi-Square test and presents by the X^2-value, degrees of freedom (df) and the p-value.

	X^2	df	p-value
3-sample test	0.169	2	0.919
2-sample test	1.064^{e}-30	1	1

Appendix 4. Significance in body mass (g) (at start) and body length (cm) between dominant individuals and subordinate individuals. These statistical analyses were conducted via a generalized linear model and presented by the estimate value, standard error (SE), z-value and p-value.

Coefficients	Estimate	SE	z-value	p-value
Mass (start)	-0.001	0.008	-0.14	0.886
Length	-0.03	0.083	-0.356	0.721

Appendix 5. Ethogram containing a code (translated to respective behavior in BORIS) for each aggressive behavior relevant for this research.

Behavior	Code	Description
Displacement	d	Aggressor approaches another fish, taking over its space (displacing). Does not pursue the displaced fish. Does not end in a bite.
Chase	c	Aggressor chases another fish. Pursues it through the observation space. Does not end in a bite.
Bite	b	Aggressor fish approaches the other fish (slowly or quickly) and closes its

		mouth on the body or fins of another fish
Mutual display	q	Two aggressing fish approach each other and line up laterally. Bodies rigid, slightly concave. Usually, the tail flexed upwards and fins standing up. Flaring gills at each other. Can be spinning slowly in a circle or can “wiggle” their bodies against each other. Coded twice (one per fish).
Mouth fighting	m	Two fish lock jaws, biting each other on their faces. Can be spinning in circles, or thrashing. Coded twice (one per fish).