How social behaviour in juvenile Brown trout (*Salmo trutta*) are regulated by social hierarchies and exposure to the benzodiazepine oxazepam

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Abstract

Improved global healthcare rises the production and consumption of human pharmaceuticals. Insufficient wastewater treatment systems and unregulated downstream impacts causes pharmaceutical contamination of surface waters with trace residues in countries covering all continents. Earlier studies of aquatic systems have shown that pharmaceutical exposure influences fish physiology and causes behavioural responses at both individual and ecosystem level. Here, I evaluated how social status in juvenile brown trout (*Salmo trutta*) influenced behaviour after exposure to an anxiolytic drug (the benzodiazepine, oxazepam). For this project, the fish were exposed to one of three oxazepam treatments: one low, environmentally relevant dose, one high, human therapeutic relevant dose, and one control dose without oxazepam. I expected decreased aggressive behaviour and mitigated relations between dominant and subordinate individuals. Contrary to the prediction and to earlier documentation of fish behavioural responses to oxazepam exposure, neither of the treatments in this project resulted in altered social hierarchies. Moreover, there were no significant differences in brown trout aggression between the treatment groups. The interspecies variations and the relatively unexplored effects of pharmaceutical exposure on social behaviour motivates further studies, preferability over longer time periods with environmentally realistic contexts. This information should be used to improve pharmaceutical regulations and legislation for ecological risk assessments.

Keywords: behaviour, brown trout, dominance, ecotoxicology, exposure, fish, oxazepam, pharmaceutical, *Salmo trutta*, social status

Sammanfattning

Sjukvården förbättras globalt vilket innebär en ökad produktion och konsumtion av läkemedel. I dagsläget är reningsverkens filtrering av avloppsvatten otillräckliga och spår av läkemedel har hittats i vattendrag över hela världen. Studier av akvatiska miljöer som kontaminerats av läkemedel har påvisat beteendeförändringar hos fiskar och andra vattenlevande organismer med konsekvenser på både individ- och ekosystemnivå. Under det här projektet utvärderade jag hur öringens (*Salmo trutta*) sociala status påverkar beteenden under exponering för ett anxiolytiskt läkemedel (bensodiazepinen oxazepam). Fiskarna exponerades för en av tre behandlingar av oxazepam: en låg dos som motsvarar koncentrationer som uppmätts i naturen, en hög och för människan terapeutiskt relevant dos, samt en kontrolldos utan oxazepam. Jag förväntade mig att exponerade fiskar skulle uppträda mindre aggressivt och att beteendeskillnader mellan dominanta och underordnade individer skulle mildras. I motsats till hypotesen och tidigare dokumentation av beteendeförändringar hos fiskar som exponerats för oxazepam resulterade ingen av behandlingarna i förändrade dominanshierarkier under detta projekt. Vidare fanns heller inga signifikanta skillnader i aggressivt beteende mellan behandlingsgrupperna. De relativt outforskade konsekvenserna av läkemedelsexponering på populations- och ekosystemnivå motiverar ytterligare studier, företrädesvis över längre tidsperioder och under miljömässigt realistiska förutsättningar. Informationen bör användas för att reglera hantering av läkemedel och vid framtida riskbedömningar av ekotoxikologiska effekter.

Nyckelord: beteende, dominans, ekotoxikologi, exponering, fisk, oxazepam, läkemedel, *Salmo trutta*, social status, öring

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1. Introduction

1.1 Aquatic systems are contaminated by human pharmaceuticals

Production and consumption of pharmaceuticals has increased in the last decades (Patel et al. 2019). Improved global healthcare has raised the demand for medical resources (van Boeckel et al. 2014) and pharmaceutical manufacturing, consumption, and disposal contributes to chemical contamination of nature. Leakage is extensive in places with inadequate disposal practices or deficient treatment facilities (Wilkinson et al. 2022), and the consequences for aquatic ecosystems are still largely unexplored (Patel et al. 2019). Wastewater effluent is a significant source of pharmaceuticals to the environment, yet conventional wastewater treatment processed do not adequately remove all pharmaceutical compounds from the effluent (Luo et al. 2014). Once in the water, fish and other aquatic animals can absorb pharmaceuticals, either directly from the water or via food intake (Mogren et al. 2013). Through biomagnification, the pharmaceuticals accumulate in the organism and could increase with each trophic level, including humans as a top predator (Kümmerer 2009). Since 2001, the European Medicines Agency requires ecotoxicological testing for pharmaceuticals entering the market (European Commission 2001/82, European Commission 2001/83), but any substances introduced earlier have not necessarily undergone ecotoxicity assessment (Martin & McCallum 2021). While pharmaceutical toxicity in humans is thoroughly studied, research about the ecotoxicological and ecological effects in aquatic environments are unrepresented (Boxall et al. 2012).

1.2 Pharmaceutical exposure can alter fish behaviour

At environmentally relevant concentrations (~1.8 µg $litre^{-1}$), human pharmaceuticals can have ecotoxicological effects on animal physiology and behaviour (e.g., Brodin et al. 2013, Hellström et al. 2016, Brodin et al. 2017, McCallum et al. 2021). In carefully balanced doses, pharmaceuticals are designed to regulate human biological functions. Therefore, exposure to human

pharmaceuticals could affect fish and other non-target organisms that have biological receptors and enzymes similar to humans (Gunnarsson et al. 2008). Pharmaceuticals such as antidepressants and other psychiatric drugs are known to affect ecologically important behaviours, such as activity, risk assessment, reproduction, and feeding rates (e.g., Kidd et al. 2006, Brodin et al. 2013, Hellström et al. 2016). Consequently, this could disrupt selective regimes and potentially lead to decreased fitness and survival. An example is oxazepam, a benzodiazepine commonly detected in freshwaters (Klaminder et al. 2015), and used to treat human anxiety, insomnia, and muscle spasms. Oxazepam acts through the gamma-aminobutyric acid (GABA) receptor, found in many vertebrate species, causing neuronal hyperpolarization and sedation (Tallman et al. 1980, Gunnarsson et al. 2008). For instance, the anxiolytic drug has been shown to disrupt behavioural endpoints in a natural population of European perch (*Perca fluviatilis*), causing fish to be more active and less social compared to non-exposed fish (Brodin et al. 2013).

1.3 The social context can influence the behavioural responses to exposure

Recent studies have mostly focused on pharmaceuticals affecting individuals (e.g., Brodin et al. 2013, Hellström et al. 2016, Brodin et al. 2017), but research about behavioural responses, mediated by the social environment in groups and populations, is scarce. Because chemical compounds can likely alter social structures, it is necessary to examine contaminants in a social context to understand their impact in a natural environment (Martin & McCallum 2021). To explore how social context modulates the consequences of a pharmaceutical exposure, McCallum et al. (2021) studied the effect of oxazepam exposure and fish social status (dominant vs. subordinate) on behaviour in juvenile brown trout (*Salmo trutta*). Exposure did not abolish the hierarchies, but the behavioural response differed depending on fish social status and oxazepam concentration. For example, subordinate fish, exposed to low concentration, became more successful competitors, compared to fish exposed to high concentration and non-exposed fish. Moreover, subordinate fish showed higher bioconcentration of oxazepam compared to dominant individuals, suggesting that social status could mediate contaminant uptake.

1.4 The aim of the project

Previous research indicates the importance of combined behavioural and toxicological studies (McCallum et al. 2021). An expected increase in the use of anxiolytics (World Health Organization 2011) motivated me to do this project, with

the aim to further investigate the effect of social context and oxazepam exposure on dominance hierarchies. Moreover, the project aimed to evaluate the repeatability of the McCallum et al. (2021) project results, using similar methods to collect and analyse data. Like for the McCallum et al. experiment, brown trout were used in this project. Juvenile brown trout are well-suited for behavioural experiments due to well-described previous research on the causes and consequences of territorial behaviour and dominance hierarchies.

This paper addressed two main issues: does oxazepam exposure affect the probability of fish changing social status (i.e., a dominant fish becomes subordinate, or vice versa), and can oxazepam exposure alter aggression? Considering the McCallum et al. (2021) results, which showed that fish absorbed different amounts of oxazepam, I predicted that exposure would change social status. Furthermore, fish that are exposed to high concentration treatment should display less aggressive behaviour compared to fish exposed to low concentration treatment or non-exposed fish.

2. Methods

2.1 Fish collection and social group formation

This project used 168 two-year old individuals of brown trout collected in the Norrfors Fish Hatchery outside of Umeå, Sweden, a site not known to receive input of pharmaceutical pollutants. The fish were brought to Umeå University, where they were housed for at least 72 hours before trials, allowing them to recover from the transport. In 1000 L tanks with ground water flow-through, the fish were fed until saturation, once a day, with both sinking and floating commercial fish pellets (INICIO, BioMar, Denmark).

During the following measurements, the fish were anaesthetised to reduce stress using MS-222 (Ethyl 3-aminobenzoate methanesulfonate; CAS number: 886-86-2; 0.1 g/L). The fish were size matched according to measurements of total length and body mass (to nearest 0.01 g) as precisely as possible and put together into groups of three. Each fish was also given a unique dorsal fin clip for identification during behavioural trials. Lastly, the fish were placed into a dark, aerated tank for sedation recovery.

2.2 Exposure and video recording

After sedation recovery, the groups of fish were transferred to a climate-controlled room and placed into glass aquaria filled with 112 L of the same groundwater as the housing tanks. The aquarium also stored a small recirculating water pump, an air stone, and a plastic plant. On day one and day seven, during each experimental replicate, water quality was monitored in two randomly selected tanks (regardless of treatment), registering dissolved oxygen concentration, temperature (YSI Ecosense), pH, conductivity, total dissolved solids, and salinity (Oakton Multiparameter Testr); nitrate, nitrite, water hardness, and chlorine (Tetra 6-in-1 multiparameter test strips). Temperature was monitored using temperature loggers (range: 9-11°C; HOBO MXtemp).

An exposure stock solution was prepared in the afternoon by dissolving oxazepam (Merck; CAS number: 604-75-1) in highly purified water. The following morning, the fish were exposed in their social groups to one of three oxazepam treatments: a low oxazepam concentration of 1.5 µg/litre, representing wastewater environment; a high oxazepam concentration of 30 µg/litre, representing a human therapeutically relevant dose; or a control treatment without oxazepam. The exposure stock solution was pipetted into the aquarium, and the water was mixed using a dip net, including a control aquarium without any stock solution. The project included five replicates, each with 10 social groups, and with at least one tank of each treatment represented. The fish were then allowed to form dominance hierarchies in their social groups. Two groups were omitted from further analysis over the course of the project due to fish mortality.

Behind opaque blinds (limiting disturbance) the fish were filmed from the side of the tank with a video recorder (2.65 MP/1080p, Sony HDR-PJ50) mounted on tripods. Over a seven-day period, each social group was recorded on four occasions for 50 minutes for each trial between 9 and 10 am, the first recording being prior to exposure and the three following past exposure. After the final recording the fish were transferred from the exposure tanks and euthanized in MS-222 (0.3 g/litre).

2.3 Scoring aggressive behaviour

After the behavioural trials, dominance and aggression were coded blind to the oxazepam exposure treatment with the computer software Boris following the ethogram established in McCallum et al. (2021) including five different dyadic social behaviours (Appendix 1). For each interaction, both the aggressor and the receiver were registered to quantify the behavioural events for each individual fish, social group, tank, and replicate. The individual fish's social status was assessed by aggression index (aggression given minus aggression received) and by position in the tank relative to the plant. A position close to the plant is coveted compared to a position restricted to the tank periphery. To standardise video lengths and to allow fish to settle after starting the camera, behaviour was scored from 10:00 minutes to 50:00 minutes (except for two videos that were cut shorter to 40 minutes).

Dominance was estimated either by aggression given or by control of the plant. On most occasions, the fish identified as dominant by aggression given was also the fish controlling the plant. When no dominant fish could be identified, all fish were assigned as “none” for dominance, i.e., non-existent aggression, or no one claimed the plant. We assume that when a fish claims the plant, it has been preceded by aggressive behaviour, while if there is aggression but none claims the plant, it implies that the dominance hierarchies are still not established. Thus, we can assign

a fish to be dominant without observing any aggressive behaviour. Consequently, to assign dominance for each day of the project, I used dominance by control of the plant, and only when no fish claimed the plant, I used dominance by aggression given.

2.4 Analysis of data

All statistical analyses were conducted using R (version 4.1.3; R Core Team 2021). Change in dominance hierarchies were analysed with a three-sample probability test (Chi-square test for equality of proportions, without continuity correction). By comparing whether the individual identified as dominant day one (prior to exposure) was replaced by another individual day six (post exposure), it was tested whether the likelihood of fish changing social status would differ between the treatments. This test required that a dominant fish could be identified both day one and day six.

Using a linear model, the effect of oxazepam treatment, time (i.e., filming day), and dominance (i.e., being assigned dominant versus subordinate) on fish aggression was analysed. Because aggression was not normally distributed, the aggression variable was log-transformed prior to analysis.

To evaluate if the average group body size differed across the oxazepam treatment groups, differences in average social group length and mass between the treatments were analysed using linear models. Further, to test if individual body size (length and mass) would predict social status (i.e., who was assigned dominant or subordinate), generalised linear models appropriate for binary response data were used.

2.5 Ethical considerations

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

3. Results

3.1 Behavioural and social consequences of exposure

I did not find any connection between oxazepam treatment and fish social status. Oxazepam exposure did not affect the probability of brown trout changing social status (estimate = 0.17, df = 2, p = 0.92) meaning that oxazepam exposure did not affect dominant fish to become subordinate (or vice versa) more often compared to non-exposed fish.

A fish exposed to high concentration of oxazepam was not differently aggressive compared to fish exposed to the low concentration of oxazepam or the non-exposed fish. Aggression given did not differ between exposed groups and non-exposed groups (control vs. low: N = 129, est. ± SE 0.0083 ± 0.042, t = -0.20, p = 0.84, control vs. high: N = 129, est. ± SE 0.015 ± 0.041, t = -0.35, p = 0.73, Figure 1).

3.2 The influence of social status and time

Dominant fish were more aggressive than subordinate fish (N = 129, est. ± SE -0.55 ± 0.036, t = -15.32, p < 0.0001, Figure 1). Further, aggression declined over the trial period (N = 129, est. ± SE -0.037 ± 0.0088, t = -4.2, p < 0.0001), indicating that social hierarchies were established.

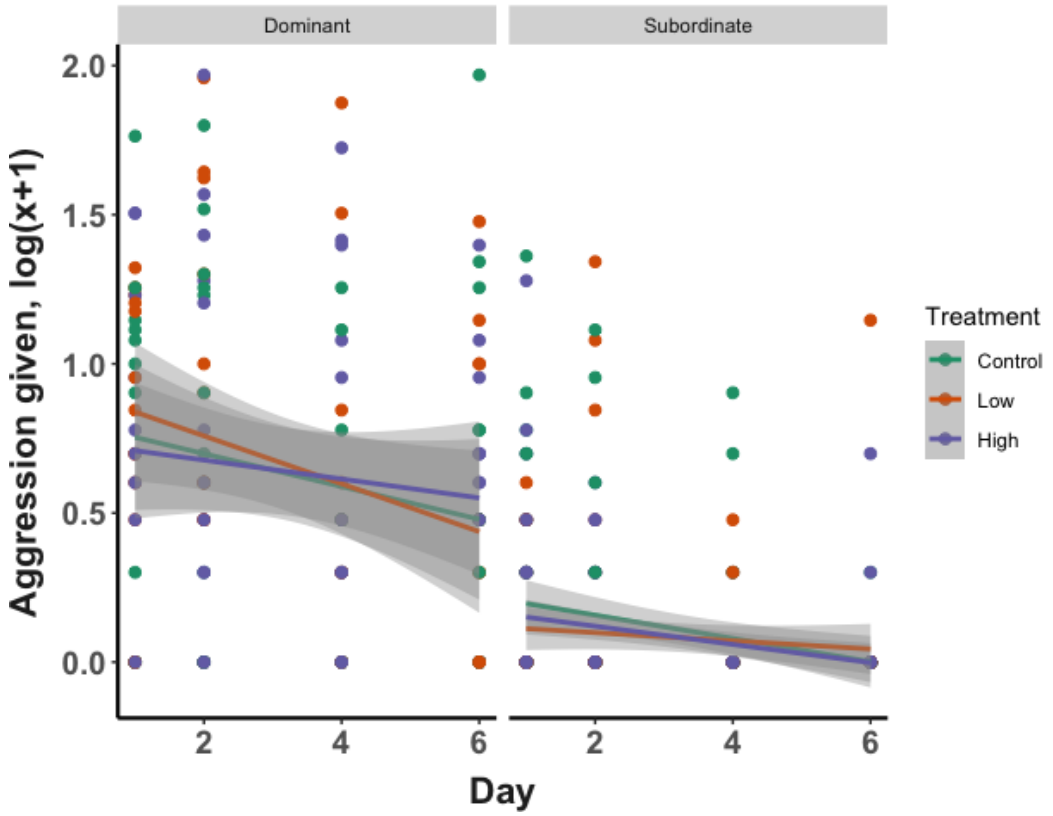

Figure 1. Dominance behaviour by treatment interactions. Aggression given (log-transformed) as response to three different concentrations of oxazepam (control: 0 µg/litre; low: 1.5 µg/litre; high: 30 µg/litre), plotted over trial, and categorised by social status. Aggression given measured as the number of expressed dyadic behaviour, following a behavioural coding ethogram (Appendix 1).

3.3 Body size impact

Average group length and mass were not significantly different among treatments (Body mass: control vs. low: N = 54, est. ± SE 0.052 ± 0.40, t = 0.13, p = 0.90, control vs. high: N = 54, est. ± SE 0.12 ± 0.40, t = 0.31, p = 0.76; Body length: control vs. low: N = 54, est. ± SE 0.97 ± 4.2, t = 0.23, p = 0.82, control vs. high: N = 54, est. ± SE 2.5 ± 4.2, t = 0.58, p = 0.56). Social status was not predicted by either body length (N = 129, est. ± SE -0.030 ± 0.083, t = -0.36, p = 0.72), or body mass (N = 129, est. ± SE -0.0011 ± 0.0075, t = -0.14, p = 0.89).

4. Discussion

4.1 Oxazepam exposure did not affect social status or aggressive behaviour

This project had two goals: to investigate how fish social status and oxazepam exposure influences dominance hierarchies, and to evaluate the repeatability of McCallum et al. (2021) results, suggesting that individual social status mediates behavioural responses to oxazepam exposure in brown trout. I predicted that exposed fish would change social status more often, and that fish exposed to high oxazepam concentrations would be less aggressive compared to non-exposed fish. Aware of the relatively small sample size, I conclude that neither of these hypotheses can be verified by data collected over this project. Earlier studies have shown that oxazepam exposure at environmentally relevant concentrations may alter fish social behaviour, although, there are interspecies variations (e.g., significant effect of oxazepam exposure: Perch—Brodin et al. 2013 and Klaminder et al. 2014, Atlantic salmon (*Salmo salar*)—Hellström et al. 2016, Roach (*Rutilus rutilus*)—Brodin et al. 2017, and Brown trout—McCallum et al. 2021, while no significant effect: Fathead minnow (*Pimephales promelas*)—Huerta et al. 2016).

It would not be far-fetched to consider body size to determine fish social status, assuming larger individuals to be dominant. If so, body size could provide the explanation, or at least a piece of the explanation, for the non-existing correlation between treatment exposure and aggressive behaviour. However, this hypothesis was rejected when neither individual or average fish length or mass were significantly different between treatment groups. Following, I suggest two other potential explanations for the absence of a relationship between treatment exposure and dyadic behaviour over this project. First, it has been shown that, at low concentrations, individuals experiencing greater predation pressure are more sensitive to oxazepam exposure (e.g., small, immature fish) compared to individuals less affected by predation pressure (e.g., larger, mature fish, Hellström et al. 2016, Hellström et al. 2020). Subordinate fish has been shown to have higher metabolic rates and cortisol levels compared to dominant fish (McCallum et al.

2021) which in turn could increase the contaminant uptake (Blewett et al. 2013). Hence, the absence of predators in a laboratory environment could explain the lack of fish social behavioural responses, suggesting that the exposure impact might vary due to ecosystems species composition (Brodin et al. 2017, Saaristo et al. 2018). Second, earlier studies have shown that brown trout can be more aggressive when observed in a laboratory environment compared to in nature which could explain why fish exposed to high concentration oxazepam treatment were as much aggressive as fish in the low dose treatment and in the control treatment (Sloman & Armstrong 2002). Further, compared to McCallum et al. (2021), this project used only three individual fish in each social group instead of four, which could decrease the competition for space in the tank and result in overall less aggressive behaviour.

4.2 Disrupted social behaviours can have consequences on an ecosystem level

Oxazepam contamination of aquatic ecosystems, altering individual fish physiology and behaviour, could modulate social hierarchies resulting in ecosystem level consequences (Kidd et al. 2006, Brodin et al. 2013, Snyder-Mackler et al. 2020). For example, increased feeding rates could provide competitive advantages at individual level (e.g., larger body mass), but at ecosystem level, intensified food intake may suppress zooplankton (i.e., fish food) which then can affect algae (i.e., zooplankton food), causing trophic cascades that affects the entire ecosystem (Brodin et al. 2013). Additionally, a fish's social status is important because the dominant fish will receive benefits by access to food, habitat, and mates (Ellis 1995) –all factors crucial for survival. As shown in McCallum et al. (2021), fish social status can, firstly, influence oxazepam uptake, and secondly, modulate the behavioural responses that are causing subordinate fish to become more competitively successful. Research combining ecology and ecotoxicology should aim to learn more about how fish social status can affect absorption of anxiolytics to better understand the responses following pharmaceutical exposure. Mitigating variations between dominant and subordinate individuals may alter selective regimes (Coe et al. 2009) and, because most fish live some parts of their lives in groups, (Snyder-Mackler et al. 2020), behavioural studies focusing on connections between individuals, populations, and ecosystems, could help to understand fish dominance hierarchies more broadly (Saaristo et al. 2018).

4.3 Challenges for future research

As pharmaceuticals become more available for the global population, it is urgent to identify the potential ecological effects for aquatic ecosystems (van Boeckel et al.

2014). Laboratory data from a simplified context might underestimate the consequences of pharmaceutical contamination in nature (Brodin et al. 2014). Some species seem to be more sensitive to exposure than others (Brodin et al. 2017), and even individuals of the same species can be differently vulnerable, depending on life stage or social status (Hellström et al. 2016, Hellström et al. 2020, McCallum et al. 2021). Also, pharmaceutical exposure causing behavioural responses could result in feedback effects, accelerating degradation of ecosystems, which further emphasises the importance of considering the social context (McCallum et al. 2021). Moreover, we must consider the variety of pharmaceuticals contaminating nature, including other types of benzodiazepines also targeting the GABA receptor, potentially underestimating the consequences that are based on a single benzodiazepine (Calisto & Esteves 2009, Boxall et al. 2012, Brodin et al. 2017). Therefore, future research should focus on identifying mechanisms and consequences of social behaviour modulated by benzodiazepines and other pharmaceuticals in aquatic systems. Further, laboratory data need to be compared with data from a realistic environment to estimate their prediction value, and to avoid underestimating the exposure effect. This could provide the information needed to predict pharmaceutical exposure effects on wildlife (McCallum et al. 2021), and to create appropriate regulations to reduce concentrations of pharmaceuticals in aquatic systems (Brodin et al. 2017, Saaristo et al. 2018).

4.4 Conclusion

The project does not provide evidence for altered social behaviour in brown trout as a response to oxazepam exposure and individual social status. This emphasises the importance to further study the causes and consequences in the variety of behavioural responses to oxazepam and other benzodiazepines in wildlife. Moreover, this enlightens the need for more complex experimental designs, with a realistic ecological environment, including interspecies relations, and performed over longer time periods.

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Appendix 1. Behavioural Coding Ethogram

Behaviour	Code	Description
AGGRESSION - dyadic		
Displacement	d	Aggressor approaches another fish, taking over its space, displacing it in space. 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. Or you can clearly see that the aggressor tries to bite the other fish but misses. You should not score two things (e.g., chase AND bite). The bite score includes the approach.
Display (rare)	q	Two aggressing fish approach each other and line up laterally or face-to-face. Bodies rigid, slightly concave, tail flexed upwards, all fins erect. Flaring gills at each other. Can be spinning slowly in a circle or can “wiggle” their bodies against each other. Code this twice, once with each fish aggressing towards the other.
Mouth fighting (rare)	m	Two fish lock jaws, biting each other on their faces, often spinning in circles or thrashing. Code this twice, once with each fish aggressing towards the other.

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