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Short Communication

Assaying waterborne psychoactive drugs by the response to naturalistic predator cues in the stickleback (*Gasterosteus aculeatus*)

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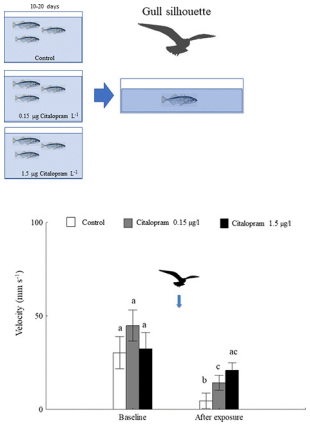
HIGHLIGHTS

- Reactions to predator cues in three-spined stickleback were evaluated.
- A passing sea gull silhouette resulted in decreased locomotor activity.
- Exposure to citalopram at ecological relevant concentrations suppressed this reaction.

GRAPHICAL ABSTRACT



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ABSTRACT

Ecotoxicological effects of psychiatric drugs and drug metabolites released by the human population are of increasing environmental concern. In this study we evaluate behavioral responses to visual predator cues in wild caught three-spined stickleback (*Gasterosteus aculeatus*) after exposure to water-born citalopram, a widely prescribed selective serotonin reuptake inhibitor with antidepressant and anxiolytic effects. Fish were exposed to ecological relevant concentrations of citalopram (0.15 or 1.5 μg L^{-1}) for 10 or 20 days. After drug exposure, individual fish were moved to a test arena where they were exposed to two naturalistic visual predator cues; a shadow from beneath, which simulated an approaching fish, and an overhead silhouette of a passing gull. Both visual cues resulted in decreased locomotor activity after post cue presentation. Notably, citalopram exposure resulted in a dose dependent suppression in response to the overhead stimulus. These results show that an ecologically relevant stimulus elicits a robust avoidance behavioral in wild caught fish after 25 min of acclimatization in the test arena. This suggests that the gull stimulus can be utilized as a behavioral endpoint in high flow through assays of ecotoxicological effects of psychiatric drugs and drug metabolites. Furthermore, the short acclimation time of wild caught fish in the test arena, opens for behavioral screening by fish living or kept in water bodies which are potentially impacted by psychiatric drugs.

1. Introduction

For most practical purposes, water pollution can be defined as an addition of something to water which alters chemical or microbial

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composition, or other properties in a way which can be harmful to either humans or animals relying on the water source, or to the aquatic organisms living in it (Lloyd, 1992). A great variety of substances and composites falls under this definition, and most sources are related to human activities which cause considerable variations in the contaminant load of water over time (Fawell and Nieuwenhuijsen, 2003). Of particular relevance to the metazoan community is the fact that about 30% of all commercially used chemicals (~30,000) may cause functional or structural changes in the nervous system of animals (Tilson et al., 1995). If released to the environment these components may have a negative impact on the ecosystem level by altering fitness related behavior (e.g. sexual behavior, predator avoidance and foraging behavior) in organisms (Hellou, 2011). In this respect, contamination of sewage treatment plant effluents, surface waters, groundwater, and drinking water by bioactive pharmaceutical substances (Fent et al., 2006) has raised concerns for aquatic wildlife (Arnold et al., 2014; Corcoran et al., 2010).

Behavior of aquatic animals is widely used for investigating the effects of contaminants in aquatic environments (Amiard-Triquet, 2009; Sievers et al., 2019). Still, utilization of studies using behavioral endpoints in regulation of chemicals is low (Ågerstrand et al., 2020) and most of the current guidelines for assessing the ecological impact of neurotoxic or neuroactive compounds are based on mammal or avian models (Legradi et al., 2018). Furthermore, several fish behavioral models used for studying the impact of bioactive substances have been translated from other vertebrate groups, e.g. rodent paradigms of human diseases (Egan et al., 2009). For example, reactions to novelty in rats and zebrafish have been used as models for studying anxiety and similar test have been used to investigate ecological effects of mood-altering drugs in fish (Kellner et al., 2016). However, that these types of models originally had a focus on human health emphasize that the ecological relevance of the species and the behavioral responses used as tests are essential for validation of the endpoint (i.e. Kane et al., 2005)

By inducing changes in anti-predator apprehensive behaviors, defined as redirected attention from activities associated with increased fitness towards detecting and/or responding to potential predators, selective serotonin (5-HT) re-uptake inhibitors (SSRIs) and other mood-altering drugs may have indirect effects on fitness (Brodin et al., 2014). In this regard, a large scale semi-natural behavioral study demonstrated that European perch (*Perca fluviatilis*) spend more time in a habitat with a higher risk of predation after exposure to oxazepam (Klaminder et al., 2016). Possible effects on other anti-predator apprehensive behaviors, such as reduced foraging or mate seeking, also suggest an ecological impact of psychiatric drugs (Brodin et al., 2014). Thus, examining behavioral responses of fish to predatory cues can potentially reveal ecological relevant effects of pharmaceuticals on ecosystems.

The aim of this study was to evaluate the potential of naturalistic predator cues in wild-caught three-spined stickleback (*Gasterosteus aculeatus*) as a behavioral endpoint for assessing the ecological impact of waterborne psychopharmaca. To do this, fish was exposed to the SSRI citalopram; a mood alter drug which is among the most frequently detected human pharmaceutical in the aquatic environment (Schultz and Furlong, 2008). Moreover, there is a growing body of evidence suggesting that environmentally relevant concentrations of these mood-altering drugs induce behavioral responses in animals. Wild caught three-spined stickleback was exposed to citalopram in ecologically relevant concentrations, whereupon their behavioral response to two different predator cues, a shadow from beneath and a passing gull silhouette, were recorded.

2. Material and methods

2.1. Experimental animals

he experiments were carried out at Flødevigen research station, Hisøya, Norway, during Aug–Sept 2016. Three-spined sticklebacks were caught with a beach seine nearby the research station. After catching, fish were kept in a flow through aquaria system in a 1.5 × 0.5 × 0.2 m (length × width × depth) holding tank for 1 week before experimentation. Fish were hand fed boiled shrimps (*Pandalus borealis*) *ad libitum* once a day. Fish weighed 0.74 ± 0.46 g (mean ± standard deviation). The water in the holding tank was unaltered local sea water with a temperature of 18–22 °C.

2.2. Test arena and protocol

A stock solution with the concentration of 3 mg L^{-1} was prepared by diluting citalopram hydrobromid in tap water and kept refrigerated under dark conditions. The stock solution was then diluted to the nominal citalopram concentrations; 0.15 and 1.5 μg L^{-1} sea water. Groups of fish were exposed to these concentrations in three 10 L aquariums for 10 or 20 days. Concentrations of citalopram of this magnitude have been identified in wastewater and are therefore considered to represent an environmentally relevant exposure scenario (Kellner et al., 2016). Control groups were exposed seawater without citalopram addition for 10 to 20 days. The aquarium water was exchanged daily and citalopram was diluted in sea water from the stock solution. Water temperature was 18–22 °C. The exposure to citalopram was performed in two rounds in the three aquaria with different concentrations of citalopram. In the first round 6 fish per treatment were exposed for 10 days, and in the second round 10 fish per treatment were exposed for 20 days. Fish were fed with boiled shrimps *ad libitum* during exposure. After exposure, single fish were moved to four test arenas (plastic aquariums 0.35 × 0.35 × 0.04 m; length × width × depth) where they were exposed to two visual cues of predation.

During netting and transfer a few fish escaped from the aquariums. Escaped individuals were excluded from the behavioral part of the study. The number of behaviorally tested fish was 5, 4 and 6 in the group exposed for 10 days, and 9, 9 and 10 in the group exposed for 20 days (at 0, 0.15 and 1.5 μg L^{-1} citalopram respectively).

The test arenas were placed on two LCD monitors (two arenas per monitor) allowing projections of predatory cues from beneath. Infrared light was reflected up to a white sheet 1 m meter above the arenas and fish were filmed with two video cameras with infra-red filters through holes in the sheet. This setup filtered the projections from beneath, allowing video tracking of fish which were undisturbed by the presentation of predatory cues from beneath. Behaviors of filmed fish were recorded and analyzed with video analysis software ethovision (Noldus Inc.).

The protocol for behavioral testing consisted of two visual challenges; a passing oval from beneath, simulating a fish predatory attack from beneath, and a gull silhouette passing above. One fish was placed in each arena and were acclimatized for 25 min before it was exposed to the predatory cue from beneath. This predatory cue was presented by a power point animation, which consisted of black ovals with centre width and length of 0.14 and 0.35 m. These ovals had velocity of 0.35 m s^{-1} and moved from the outside of the arenas until they were completely under the arenas, whereupon they returned. Each fish was presented to the oval five times, with a two second pause in between (see Supplementary material 1). Five minutes after the predatory cue from beneath, fish were exposed to a gull silhouette which was sliding at an approximate velocity of 0.5 m s^{-1} on fishing lines 0.8 m above the arenas (see Supplementary material 2).

2.3. Behavioral and data analysis

The behavioral responses to the visual predator attacks were analyzed by locomotor activity (mm/s) 20 s before (baseline) and after the simulated predator attacks. In addition, locomotor activity during the exposure to an oval from beneath was analyzed. Locomotor activity was log transformed to obtain normal distribution and, thereafter analyzed with repeated measure two-way analysis of variance (ANOVAs), with treatment and exposure times as independent variables. Significant differences were further investigated with the unequal N HSD

post hoc test. Data are presented as means ± standard error of the mean (SEM) if not otherwise stated.

3. Results

3.1. Shadow from beneath

There was a general effect of exposure to the shadow from beneath (ANOVA $F_{(2, 74)} = 12.7$, $P < 0.001$). Locomotor activity after exposure to the predator cue was significantly lower than values before ($P < 0.001$) or during ($P < 0.001$) predator exposure (Fig. 1). Moreover, there were no significant differences between locomotor activity before and during the predator exposure ($P = 0.8$). However, there were not any effects of citalopram treatment (ANOVA $F_{(1,37)} = 0.09$, $P = 0.73$), exposure time ($F_{(1,37)} = 1.43$, $P = 0.24$) or interaction effects between these factors (ANOVA $F_{(4,74)} = 1.1$, $P = 0.43$).

3.2. A passing gull silhouette

Citalopram treatment significantly affected locomotor activity before and after exposure to a gull silhouette (ANOVA $F_{(2,37)} = 5.6$, $P < 0.01$). Specifically, control ($P < 0.001$) and 0.15 μg L^{-1} citalopram treatment resulted in a significant decrease ($P < 0.005$) in locomotor activity, while this effect was not present in the group treated with 1.5 μg L^{-1} citalopram ($P = 0.16$) (Fig. 2). This dose dependent effect in locomotor activity was also evident after gull exposure. Control fish had lower locomotor activity than the group treated with 0.15 μg L^{-1} citalopram ($P < 0.05$), and the group treated with 1.5 μg L^{-1} citalopram ($P < 0.001$). Moreover, there were no significant differences in locomotor activity between the group treated with 0.15 μg L^{-1} and the group treated with 1.5 μg L^{-1} citalopram ($P = 0.16$) (Fig. 2). Furthermore, there were no significant differences between treatment groups before exposure to gull exposure ($P < 0.99$). This effect was independent of exposure time (ANOVA $F_{(2,37)} = 0.67$, $P = 0.42$), and there were no interactions between exposure time and citalopram treatment (ANOVA $F_{(2,37)} = 0.64$, $P = 0.53$).

4. Discussion

This study demonstrates a dose responsive suppression in behavioral reaction to a passing gull silhouette following exposure to citalopram. The drug induced effect was less expressed when fish were exposed to a shadow from beneath.

Generally, fish showed an increase in locomotor activity when exposed to shadow from beneath. This response most probably reflected a predator escape behavior. That this response tended to be suppressed and that the avoidance behavior to the passing gull silhouette was suppressed by citalopram is in accordance with biomedical fish models showing anxiolytic effect of SSRIs (Connors et al., 2014). For example, the novel diving test, a zebrafish model of anxiety where the behavioral response to being placed in a novel aquarium is quantified, has demonstrated anxiolytic effects of the SSRI fluoxetine (Stewart et al., 2014). Moreover, Kellner et al. (2016), demonstrated that this model could also be utilized for detecting effects of citalopram in environmentally relevant concentrations in three-spined sticklebacks. In addition, drug induced effects on the time taken to approach an unfamiliar object was also reported in the latter study. In general, behavioral responses to novelty, or neophobic reactions, have been associated with fitness related traits such as avoidance to novel predators and willingness to utilize new feed sources (Greggor et al., 2015). In line with this, Kellner et al. (2015) suggested ecological consequences of citalopram at concentrations of 1.5–15 μg L^{-1}. In the present study, citalopram reduced the decrease in locomotor activity, elicited by the passing gull silhouette, in a dose dependent manner. Thus, our results demonstrating a suppressed response to a naturalistic predator stimulus together with other studies, which generally show a suppressive effect on predator avoidance (Dielenberg and McGregor, 2001; Pelli and Connaughton, 2015), lends further support to ecological impacts of SSRIs.

In the present study, the response was observed in wild caught three-spined sticklebacks after just 25 min of acclimatization in the test arena which suggests that it is robust enough for being used as a high throughput behavioral endpoint in ecotoxicological assay. The ecological relevance of this behavioral endpoint is further strengthened by the fact that three-spined sticklebacks inhabit a wide variety of freshwater, brackish seashore and estuarine areas (Froese, 2017), and are an important prey species (Reimchen, 1994). Thus, potentially, ecological effects can be detected by changes in behavioral reaction to the passing gull silhouette in sticklebacks that have been living or kept in water bodies which are impacted by psychiatric drugs. However, it is important to point out that in the current study fish were kept in a holding tank one week before experimentation. This stresses that further studies, including investigating potential effects of the acclimation time from catch to testing, are needed for making the current endpoint applicable in fish that have been kept in water bodies with potential behavioral altering contaminants. Moreover, in a recent review by Legradi

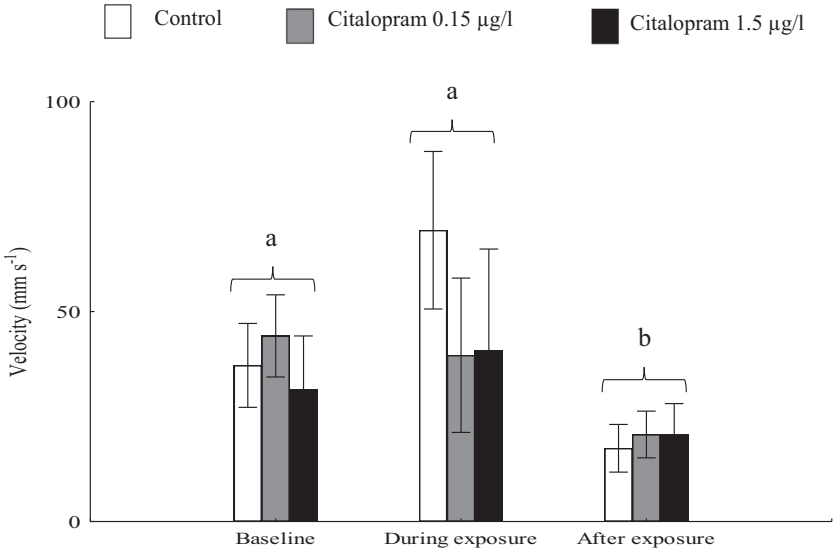

Fig. 1. Locomotor activity in three-spined sticklebacks 20 s before, during and 20 s after exposure to a shadow from beneath. Before exposure to this visual predatory cue fish were exposed to citalopram at different concentrations for 10 and 21 days. Results are from a two-way repeated ANOVA with treatment time and citalopram concentrations as independent variables. Letters indicate significant differences between different time intervals at the level $P < 0.05$. For further statistical information, see results and material and methods.

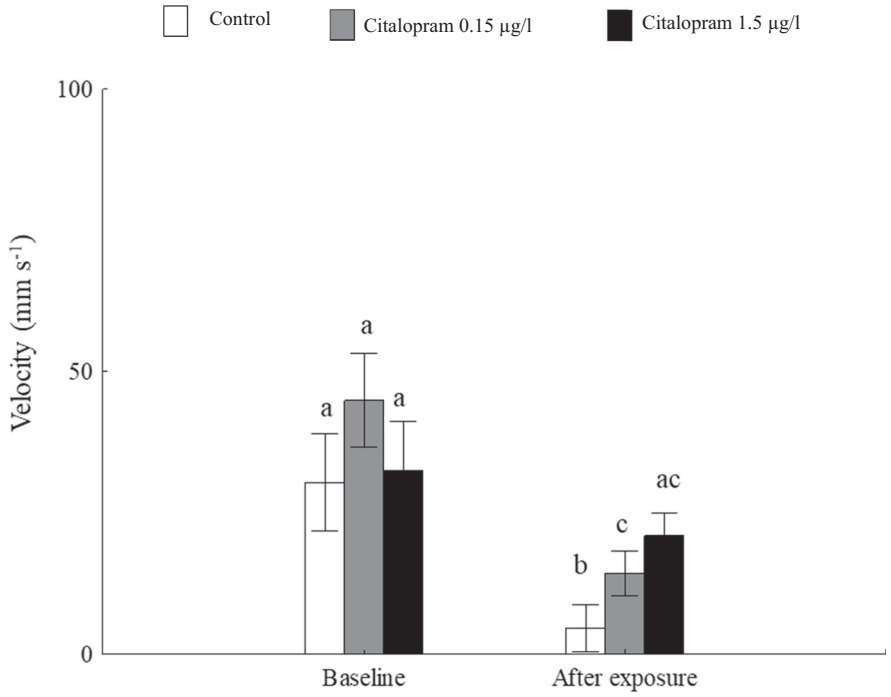

Fig. 2. Locomotor activity in three-spined sticklebacks before and after a passing gull silhouette. Before exposure to this visual predatory cue fish were exposed to citalopram at different concentrations for 10 and 21 days. Letters indicate significant differences between different time intervals at the level $P < .05$. Results are from a two-way repeated ANOVA with treatment time and citalopram concentrations as independent variables. For further statistical information, see results and material and methods.

et al. (2018) it was pointed out that the number of potential neurotoxic or neuroactive compounds in the environment are raising and that behavioral assays with target species within the ecosystem together with *in situ* and *in silico* methods are needed to assess the environmental hazards of these compounds. Considering the ecological relevance of the three-spined sticklebacks, we suggest that the predator avoidance in response to the simulated bird attack could be an integrated part in investigations of the environmental impact of substances with potential neurotoxic or neuroactive effects.

5. Conclusions

Here we report that a visual predator cue in the form of a gull silhouette passing overhead resulted in a prompt decrease in locomotor activity in wild caught three-spined sticklebacks. This response was robust, present after just 25 min of acclimation to the novel test arena, and was suppressed by citalopram at environmentally relevant concentrations, demonstrating the potential usefulness of this model in high throughput assays for other substances.

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CRediT authorship contribution statement

Erik Höglund: Writing - original draft, Investigation, Formal analysis, Conceptualization. **Øyvind Øverli:** Data curation, Writing - original draft. **Åse Åtland:** Methodology, Data curation, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Anima ethics

The experimental work in this study was approved by the Norwegian Food Safety Authority. License number 8635.

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