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Natural daily patterns in fish behaviour may confound results of ecotoxicological testing☆

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ABSTRACT

Low doses of neuroactive chemicals end up in the environment and disrupt behaviour of non-target organisms. Although a whole range of studies have documented pollutant-induced changes in behaviour, natural daily variability in behaviour is rarely taken into account. This is surprising because biological rhythms may affect the outcome of experiments, are adaptive and are expected to be sensitive to neurochemical exposure. Here, we exploit daily behavioural variation in the fish model *Nothobranchius furzeri* to examine if behavioural effects of chronic exposure (74 days) to an environmentally relevant level (28 ng/L) of the neurochemical fluoxetine depend on the time of day. Fluoxetine exposure induced an increase in anxiety-related behaviour that was slightly more pronounced in the evening compared to the morning. Moreover, open-field locomotor activity was disrupted and daily patterns in activity lifted upon exposure to the compound. These results imply that short-term behavioural variability should be considered both to standardise ecological risk assessment of neuroactive chemicals as well as to better understand the environmental impact of such compounds in aquatic ecosystems.

1. Introduction

The introduction of synthetic chemicals into natural environments is a major contributor to global environmental change (Steffen et al., 2015; Waters et al., 2016). Over the past four decades, the rate at which a wide diversity and quantity of chemicals was produced and released into ecosystems has been unparalleled (Bernhardt et al., 2017). Many compounds including pesticides and pharmaceuticals adversely affect non-target species in various ways and threaten the integrity of ecosystems (Besson et al., 2020; Brodin et al., 2013).

While substantial efforts are made to keep the presence of these chemicals in the environment below lethal levels (Brady et al., 2017), various sub-lethal effects may still indirectly affect survival and population fitness (Melvin et al., 2016). This is especially true for neuroactive chemicals such as antidepressants and anxiolytic pharmaceuticals that are typically present in the environment in the mid to low ng/L range (Puckowski et al., 2016). One example is fluoxetine, the active compound of Prozac and one of the most prescribed neuroactive drugs (Kwon and Armbrust, 2006), which often occurs in surface waters at concentrations < 600 ng/L (Puckowski et al., 2016; Saaristo et al., 2017). Such concentrations seem negligible, yet neuroactive chemicals are highly potent and act already at low doses on evolutionarily well-conserved physiological pathways of non-target species (Melvin, 2017).

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and, in humans, directly acts on the serotonergic system by binding to serotonin transporters in nerve endings. This inhibits the reuptake of the neurotransmitter serotonin in the synaptic cleft and leads to higher levels of extracellular serotonin (Kellner et al., 2015; Mcdonald, 2017). In fish, the serotonergic system controls various physiological and behavioural systems (reviewed by Mcdonald, 2017), and SSRI-induced changes in fish serotonin level may cause comparable target-mediated effects in fish as in humans (Margiotta-Casaluci et al., 2014).

Over the years, a vast amount of information accumulated on how neuroactive chemicals affect wildlife through specific biological effects, especially with regard to changes in behavioural expression (Melvin, 2017; Sumpter et al., 2014). For instance,

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exposure of fish to different doses of fluoxetine affects locomotion and feeding behaviour, as well as behaviour related to stress, anxiety and aggression (Mcdonald, 2017). Although integrating such information should show the risks of neuroactive chemicals for wildlife at sub-lethal doses, reaching any evidence-based consensus is hampered because reported results are hardly unanimous in terms of sensitivity to compounds (Melvin, 2017; Sumpter et al., 2014). Likely, methodological differences between studies partly underlie the observed discrepancies (Melvin, 2017; Sumpter et al., 2014). This implies overlooked variables that influence the toxicity outcomes of neurochemical exposure. It is not only important to identify these factors to standardise ecotoxicological tests for neurochemicals, but also to accurately estimate the environmental impact of neurochemicals.

Classic ecotoxicological assessment of acute exposure effects is increasingly criticized for not reflecting realistic scenarios and its inability to ascertain effects of long-term exposure (Thoré et al., 2020; 2021b). This is especially relevant for many pharmaceuticals that persist in the environment due to their low biodegradability and continuous discharge (Kwon and Armbrust, 2006; Puckowski et al., 2016). Fluoxetine, for instance, is relatively resistant to hydrolysis, photolysis and microbial degradation, and has a half-live that exceeds 100 days in aqueous solutions (Kwon and Armbrust, 2006). Nevertheless, even studies that adopt similar exposure regimes over more ecologically-relevant time windows often still reach different conclusions with regard to behavioural responses to pollutants. For instance, adult mosquitofish (*Gambusia holbrooki*) exposed for 28 days to 25 ng/L of fluoxetine were shown to exhibit increased activity levels (Martin et al., 2017). Yet, in another study, a similar exposure regime of 31 ng/L over 35 days did not elicit any effects on activity (Martin et al., 2019a).

Besides variability in length of exposure, it may also be important to consider that animals often display natural biological rhythms, such as daily fluctuations in behaviour (Bulla et al., 2016; Patke et al., 2020). To date, however, very few attempts have been made to explicitly assess its importance for ecotoxicological testing (Prokkola and Nikinmaa, 2018; Zhao and Fent, 2016). This is surprising for at least three reasons. Firstly, it is common knowledge in behavioural sciences that daily fluctuation in physiology and behaviour may affect the outcome of experiments and should be controlled for by sampling at the same time points (Prokkola and Nikinmaa, 2018). Secondly, daily fluctuation in behaviour may have high adaptive value (Prokkola and Nikinmaa, 2018; Westwood et al., 2019) and any changes may affect a whole range of fundamental ecological interactions including predator-prey (Bulla et al., 2016) and parasite-host (Westwood et al., 2019) interactions. Thirdly, many neuroactive chemicals likely affect daily fluctuations in behaviour. For instance, neurotransmitters such as serotonin control the sleep/wake cycle in humans (Portas et al., 2000), and SSRI treatment directly affects circadian rhythms (Walker II et al., 2020). Likewise, serotonin may regulate circadian rhythmicity and associated behaviour in fish (Kreke and Dietrich, 2008), and exposure to fluoxetine impairs circadian rhythm signalling in zebrafish (*Danio rerio*) (Vera-Chang et al., 2018).

To examine the relevance of natural biological rhythms for ecotoxicological testing in controlled laboratory conditions, we assess the impact of chronic fluoxetine exposure at an environmentally relevant concentration in relation to daily behavioural variability in the turquoise killifish *Nothobranchius furzeri*. In specific, as *Nothobranchius* killifish exhibit stable daily locomotor rhythms (Lucas-sánchez et al., 2013; Lucas-Sánchez et al., 2014) with consistent higher levels of activity in the morning than the evening (Thoré et al., 2019), we assess how fluoxetine exposure may affect anxiety-related behaviour and open-field activity on different moments of the day (morning vs. evening). Previous research showed that *N. furzeri* behaviour is sensitive to fluoxetine exposure (Thoré et al., 2018b, 2020), and based on a cross-species extrapolation approach in which comparable target-mediated effects of fluoxetine exposure are hypothesised, we expect that the impact of chronic fluoxetine exposure on activity and anxiety will depend on the time of day.

2. Materials and methods

2.1. Study species and fish maintenance

Experiments were performed from January 22, 2018 to April 27, 2018, using *N. furzeri* as study species. This annual fish species inhabits temporary freshwater ponds (rainy season) in south-east Africa, and survives the periodic drying of their habitat (dry season) by producing drought-resistant eggs that remain dormant in the sediment (Cellerino et al., 2015). At the onset of the next rainy season, hatchlings rapidly grow and reach sexual maturity in <3 weeks (Polačik et al., 2016). Mainly owing to its fast life-history, the species rapidly gained popularity as a model species for several fields of research, including ecotoxicology (Thoré et al., 2021a).

94 fish of the homozygous laboratory GRZ-AD strain were hatched by submerging ready-to-hatch eggs (Stage 43, sensu Wourms, 1972) in reconstituted water (Instant Ocean Salt mix added to type III RO water to a conductivity of 600 μS/cm) with 1 g/L humic acid (53,680; Sigma-Aldrich) (cf. Philippe et al., 2018). Hatchlings were kept in 2.5 L tanks (18 cm long x 12 cm wide x 11.5 cm high) at a maximum density of 15 fish/2 L until 21 days post hatching (dph), after which fish were individually transferred to transparent 2.5 L tanks under static conditions (18 cm long x 12 cm wide x 11.5 cm high) for the remainder of the experiment. This setup facilitated individual monitoring while allowing visual contact between fish and limiting agonistic behaviour to ensure animal welfare. Hatchlings were fed twice a day to satiation with *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium) until 35 dph. After that, fish were fed once a day (at 9.00pm to standardise the feeding time) to satiation with frozen *Chironomus* larvae (Ocean Nutrition, Essen, Belgium). Until 21 dph, any excess food was removed from the tanks on a daily basis using a pipet to maintain good water quality. Starting from 21 dph and for the remainder of the experiment, tanks were cleaned twice a week (complete water renewal on Monday and Thursday) and water quality was monitored (7.8 pH, ammonium <0.2 mg/L, nitrate < 25 mg/L, nitrite <0.2 mg/L). Throughout the experiment, fish tanks were kept in a temperature-controlled system to ensure a constant water temperature of 24 °C, under a 14 h light: 10 h dark photo-regime (lights were switched on and off at 8.00am and 10.00pm, respectively). Light was provided at a constant light intensity of 2000 lux (full spectrum white LED light) at fish level.

2.2. Experimental setup and behavioural testing

At 21 dph, fish were randomly assigned to one of two experimental conditions: a control condition (n = 48) and a condition in which fish were continuously exposed for a total of 74 days (i.e., until 95 dph) to a nominal dose of 50 ng/L fluoxetine hydrochloride (F-132; Sigma-Aldrich) (n = 46)(Fig. 1). This nominal fluoxetine concentration was chosen to represent an environmentally relevant level of the compound in surface waters (Martin et al., 2019b).

Starting at 57 dph (i.e., 36 days after the start of the fluoxetine treatment) and until 94 dph (Fig. 1), fish behaviour was recorded twice a day (morning between 9.00 and 12.00am and evening

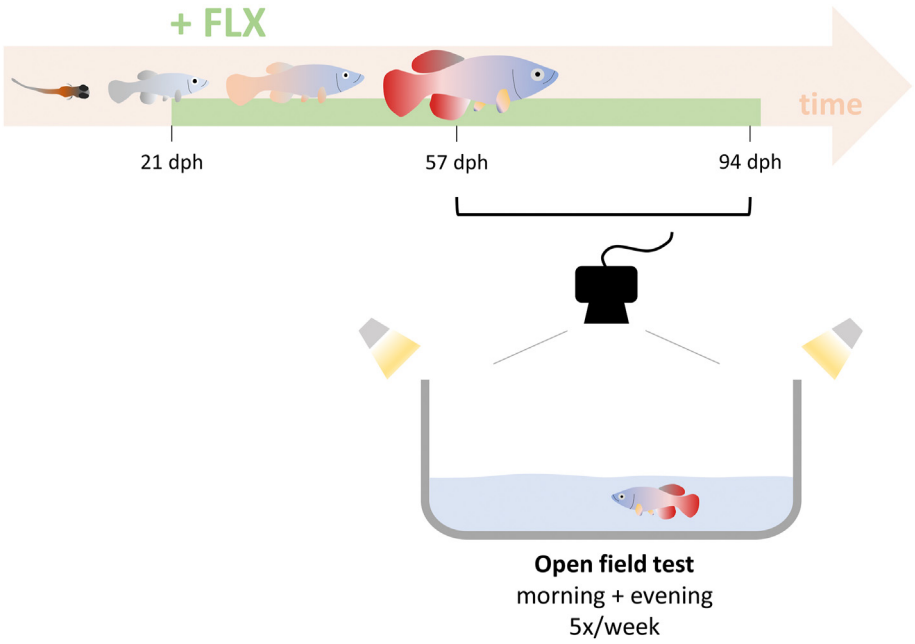

Fig. 1. Experimental setup. Starting 21 days post hatching (dph), *N. furzeri* fish were chronically exposed to 25 ng/L fluoxetine. Starting 57 dph and until 94 dph, individual fish were subjected to an open-field test in the morning and the evening to assess activity level and anxiety-related behaviour, five times per week.

between 6.00 and 9.00pm) for five subsequent days per week by means of an open-field assay. In doing so, a total of 27 repeated measures were obtained for each individual. For each trial, fish were individually transferred to a test arena (18 cm long x 12 cm wide x 11.5 cm high) with a water level of 2 cm (water volume of 0.5 L) to confine the fish to primarily two dimensional movement. Before starting each trial, fish were allowed to acclimate for 5 min (cf. Thoré et al., 2018b; 2018a). Subsequently, fish were filmed (top view) for 15 min using Logitech C920 HD Pro webcam digital cameras. Test arenas had a white base to ensure sufficient contrast between the fish and their background, and grey opaque screens were positioned around each arena to prevent confounding social interactions between individuals. After each trial, fish were transferred back to their respective housing tanks. Video-recordings were analysed afterwards (observer-blind) using EthoVision XT Version 9.0 video-tracking software (Noldus Information Technologies, Wageningen, the Netherlands).

The open-field set-up is commonly used to assess both fish locomotor activity and anxiety-related behaviour (Ansai et al., 2016). In this set-up a range of behavioural traits can be assessed without the need for further manipulation. Specifically, travelled distance (cm), calculated as total distance moved of the centre-point of the fish, and total moving time (sec), calculated as the duration for which the centre-point of the fish was changing location, with thresholds at 2.00 (start) and 1.75 (stop) cm/s, were assessed as measures for open-field activity level. In addition, also average swimming velocity (cm/sec) and maximum acceleration (cm/sec^2) were assessed. Typically, activity in the periphery of the arena ('thigmotaxis') reflects an anxiety-like state whereas activity in the centre of the arena is considered risk-prone behaviour (Ansai et al., 2016; Godwin et al., 2012). Here, total distance (cm) between the centre point of the fish and the centre of the arena (calculated per video frame and summated over the 15min duration of the test) was assessed as a measure for anxiety-related behaviour.

At 95 dph (i.e., the day after the last open-field trial), total body length (dorsal view from the tip of the snout to the tip of the tail) was determined for each individual. To this end, each fish was temporarily transferred to a petri-dish with a small amount of water that is sufficient for the fish to sustain a natural dorso-ventral posture. Fish were centred in the frame (dorsal view) of a Samsung Galaxy S8+ dual-pixel 12.0 MP AF F/1.7 camera upon obtaining size-calibrated photographs that were analysed digitally using the open source image processing software ImageJ Version 1.50i (Schneider et al., 2012).

2.3. Preparation of solutions

A 500 mg/L fluoxetine hydrochloride (Sigma F-132) stock solution was prepared using dimethyl sulfoxide (DMSO) as a solvent, and was subsequently stored at −20 °C as aliquots. When needed, working solutions were prepared by diluting stock solution aliquots with 600 μS/cm reconstituted water to a concentration of 5 mg/L. Working solution was added to the tanks to a nominal concentration of 50 ng/L. Throughout the experiment, water samples were taken at random before renewal of the water and analysed using liquid chromatography coupled to mass spectrometry (LC/MS/MS) to analyse for the presence of fluoxetine hydrochloride. The mean detected level of fluoxetine in the samples was 28.2 ng/L (STDEV: 16.9 ng/L, n = 13). As DMSO was used as a solvent to prepare fluoxetine solutions, control fish were exposed to an equal amount of DMSO (0.1e-5%) as fish from the fluoxetine condition. Treatments were applied each time when the water was renewed (twice per week, see above).

2.4. Animal welfare note

All experimental procedures and methods conform to the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P070/2016). Two researchers independently (E. S. J. Thoré and B. De Rijck) monitored the condition and health of each individual fish for a minimum of two times per day. Optimal water conditions were provided, and water quality was measured twice a week (7.8 pH, ammonium <0.2 mg/L, nitrate < 25 mg/L, nitrite <0.2 mg/L). Any disturbance

and handling that was not strictly necessary for the experiment was kept to a minimum to prevent and limit stress.

2.5. Statistical analyses

All statistical analyses were performed in R 3.3.1 (R Development Core Team, 2016) at a significance level of alpha = 0.05. For all analyses, the model assumptions including distributional fit and homogeneity of variances were verified graphically, complemented with a Shapiro-Wilk test for normality.

Travelled distance, total moving time, average swimming velocity, maximum acceleration and total distance between the fish and centre of the arena were analysed by means of linear mixed models with Gaussian error distribution (lme4 package, Bates et al., 2017). Time (morning, evening), condition (control, fluoxetine) and sex (male, female) were included as fixed factors, including their full interaction. In addition, also day (referring to the consecutive 37 days of the observation period between 57 and 94 dph, Fig. 1) and fish body length were added as fixed factors. Fish identity was added as a random effect to the models.

To test the significance of the fixed effects, parametric bootstrapping with 1000 simulations was used (afex package, Singmann et al., 2017). Post-hoc differences were assessed by means of Tukey-corrected pairwise comparisons (lsmeans package, Lenth and Love, 2017).

3. Results

Travelled distance was higher in the morning (mean ± SE 1648.776 ± 48.928 cm) than in the evening (mean ± SE 1549.175 ± 48.963 cm) (Tukey $P <$ **0.001**), however only for fish that were not exposed to fluoxetine (Table 1, Fig. 2A). Fish that were exposed to fluoxetine travelled a smaller distance than control fish (Table 1, Fig. 2A) and showed no difference in activity level in the morning (mean ± SE 1334.093 ± 49.980 cm) compared to the evening (mean ± SE 1343.909 ± 50.010 cm) (Tukey $P = 0.976$). In addition, travelled distance differed between days and overall increased with age (Table 1, Fig. S1), with fish traveling more than double the distance on the last day (day 27, mean ± SE 1725.040 ± 51.019 cm) of behavioural observation compared to the first day (mean ± SE 755.314 ± 50.608 cm). All results for total moving time followed a similar pattern (Table 1, Fig. 2B).

Control fish also swam at a higher mean velocity in the morning (mean ± SE 1.856 ± 0.056 cm/s) compared to the evening (mean ± SE 1.746 ± 0.056 cm/s) (Tukey $P <$ **0.001**). Fish that were exposed to fluoxetine swam at a lower mean velocity than control fish (Table 1, Fig. S2) and their mean velocity in the morning (mean ± SE 1.521 ± 0.057 cm/s) did not differ from that in the evening (mean ± SE 1.525 ± 0.057 cm/s) (Tukey $P = 0.999$). Mean swimming velocity differed between days and increased with age (Table 1, Fig. S3), with fish swimming at a velocity that was more than double on the last day (mean ± SE 1.992 ± 0.059 cm/s) of behavioural observation compared to the first day (mean ± SE 0.858 ± 0.058 cm/s).

Maximum swimming acceleration was higher for males (mean ± SE 3.981 ± 0.023 cm/s^2) than females (mean ± SE 3.718 ± 0.023 cm/s^2) (Table 1). In addition, fish had a slightly higher maximum swimming acceleration in the morning (mean ± SE 3.868 ± 0.018 cm/s^2) compared to the evening (mean ± SE 3.842 ± 0.019 cm/s^2) (Table 1). Fish exposed to fluoxetine (mean ± SE 3.979 ± 0.023 cm/s^2) showed a higher maximum acceleration than control fish (mean ± SE 3.737 ± 0.023 cm/s^2) (Table 1, Fig. 2C). Maximum swimming acceleration varied amongst days but did not follow a clear trend over time (Table 1, Fig. S4).

Total distance to the centre of the arena was lower in the morning (mean ± SE 35987.370 ± 212.080 cm) than in the evening (mean ± SE 36647.320 ± 212.354 cm) (Tukey $P <$ **0.001**), however only for fish that were exposed to fluoxetine (Table 1, Fig. 2D). Fish that were not exposed to fluoxetine swam closer to the centre of the arena than fluoxetine-exposed fish (Table 1, Fig. 2D) and showed no difference in distance to the centre in the morning (mean ± SE 33013.680 ± 207.569 cm) compared to the evening (mean ± SE 33199.220 ± 207.891 cm) (Tukey $P = 0.495$). In addition, total distance to the centre of the arena differed between days and tended to increase with age (Table 1, Fig. S5), with an approximate increase of 10% in total distance to the centre between the first (mean ± SE 30942.44 ± 272.218 cm) and the last day (day 27, mean ± SE 35217.80 ± 275.141 cm) of behavioural observation.

4. Discussion

To date, short-term variability in behaviour has been largely overlooked as a potential mediator and sensitive endpoint of pollutant impact on wildlife. In the current study, we exploit daily behavioural variation in the fish model *N. furzeri* to examine how chronic exposure to an environmentally relevant level of the neurochemical fluoxetine may affect fish behaviour with relation to the time of day. The results show that exposure to fluoxetine increases anxiety-related behaviour, with a slightly higher effect in the evening (+1.8%) compared to the morning. Moreover, fluoxetine reduces open-field locomotor activity and lifts daily activity patterns. Overall, our findings may offer a partial explanation for the often divergent behavioural effects of chemical exposure in ecotoxicological studies.

Table 1
Variation in open-field activity (travelled distance, total moving time, mean swimming velocity, maximum acceleration) and anxiety-related behaviour (total distance to centre) was analysed using linear mixed models.

	Travelled distance		Total moving time		Mean velocity		Max. acceleration		Total distance to centre	
Effect	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value
Time	9.320	**0.007**	7.426	**0.006**	9.326	**0.007**	8.958	**0.004**	21.149	**0.001**
Condition	13.593	**0.001**	13.479	**0.001**	12.003	**0.002**	33.458	**0.001**	83.159	**0.001**
Sex	1.634	0.214	3.536	0.061	1.029	0.348	43.838	**0.001**	2.424	0.145
Day	1686.690	**0.001**	1980.242	**0.001**	1607.624	**0.001**	281.777	**0.001**	1121.994	**0.001**
Body length	0.559	0.468	0.524	0.480	0.667	0.432	<0.001	1.000	0.067	0.798
Time*Condition	13.264	**0.001**	17.141	**0.001**	10.582	**0.001**	1.219	0.273	6.876	**0.012**
Time*Sex	2.144	0.154	2.607	0.101	2.095	0.151	0.619	0.415	2.049	0.154
Condition*Sex	0.318	0.572	0.621	0.435	0.71	0.564	1.226	0.266	0.257	0.628
Time*Condition*Sex	0.003	0.956	0.032	0.852	0.001	0.968	0.232	0.631	2.810	0.087

P-values < 0.05 are shown in bold.

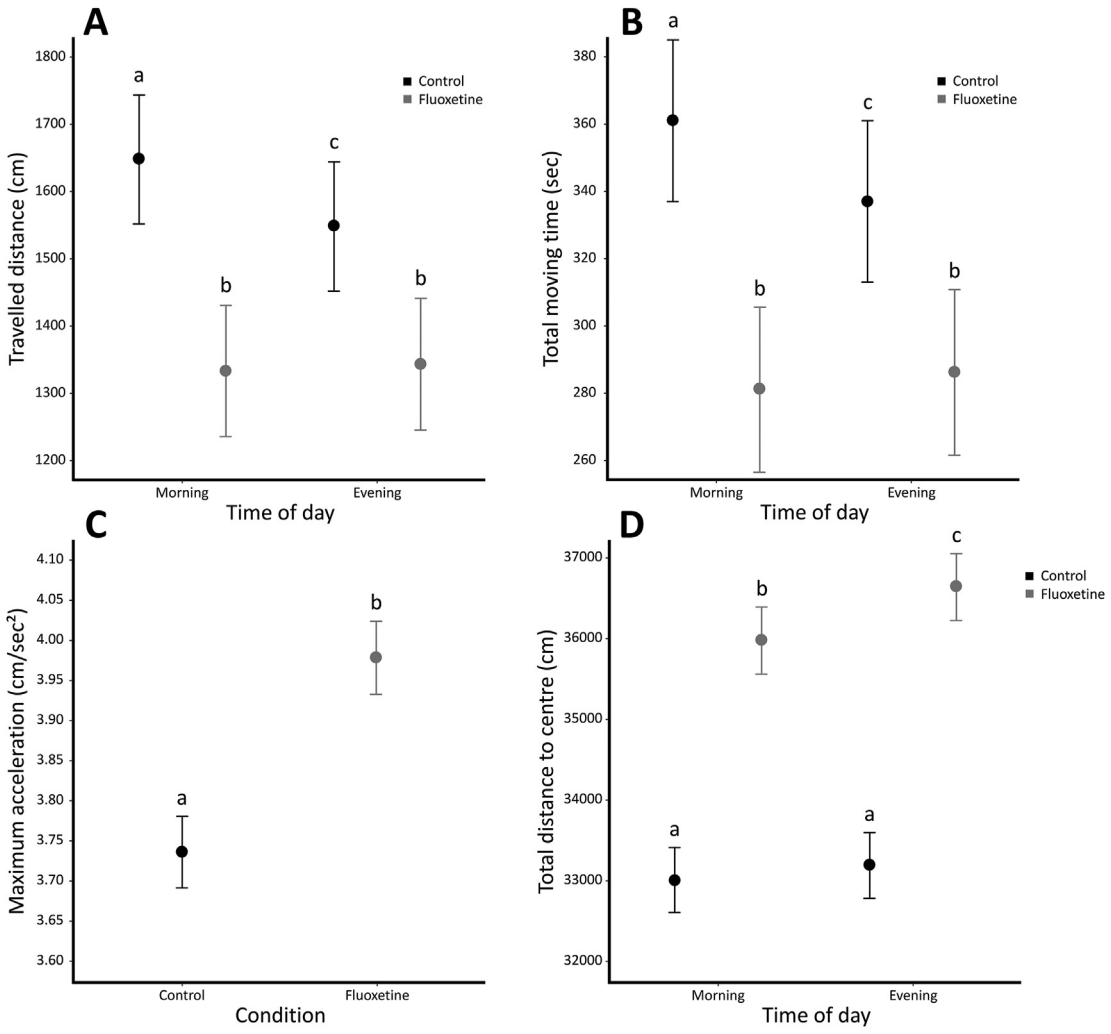

Fig. 2. Open-field activity and anxiety-related behaviour in *N. furzeri* with relation to fluoxetine exposure and daily fluctuation in behavioural expression. (A) Total travelled distance (cm) and (B) total moving time (sec) as a measure for open-field activity level, (C) maximum acceleration (cm/sec^2), and (D) total distance to the centre of the test arena (cm, cumulative distance per frame) as a measure for anxiety-related behaviour during a 15-min open-field test for control fish and fish exposed to 50 ng/L fluoxetine (nominal concentration) with relation to time of day. Differences in behavioural expression between the morning and evening were not found for maximum swimming acceleration and are therefore not included in the figure. Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests.

4.1. Fluoxetine exposure increases anxiety-related behaviour and inhibits activity

As predicted by serotonin-neurophysiology, chronic fluoxetine exposure disrupted anxiety-related behaviour and locomotor activity in *N. furzeri*. In teleost fish, a whole range of behavioural traits are to a large extent controlled by the hypothalamic-pituitary-interrenal (HPI) axis through secretion or inhibition of various (neuro)hormones (Mcdonald, 2017). SSRI-induced elevation of extracellular serotonin levels regulates the HPI axis at all levels and instigates a cascading effect of intricately-related hormones that control behaviour. Principally, serotonin stimulates the release of corticotropin releasing hormone from the hypothalamus which, in turn, stimulates the release of adrenocorticotropin at the level of the pituitary gland (Mcdonald, 2017; Mommsen et al., 1999). This further stimulates the release of the stress hormone cortisol from the kidney interrenal cells, which ultimately regulates the transcription of a range of genes including those involved in centrally-mediated behaviours (Mcdonald, 2017; Takahashi and Sakamoto, 2013). Cortisol further exerts a negative feedback on both the release of hormones of the HPI axis and on the number of serotonin-receptors in the brain, thereby attenuating their involvement along the HPI axis (Medeiros et al., 2014; Mommsen et al., 1999). In addition to their involvement in the HPI axis, serotonin-receptors are also present in other brain regions where they mediate changes in anxiety (Jesuthasan, 2012; Mcdonald, 2017). Stimulation of these serotonin-receptors results in less anxiety-related behaviour (anxiolytic effects), whereas inhibition of such receptors may result in both less or more (anxiogenic effect) anxiety-related behaviour (Mcdonald, 2017).

In fish, exposure to SSRI's including fluoxetine generally results in anxiolytic effects (Mcdonald, 2017; Saaristo et al., 2017). However, also opposite results are reported, likely in part due to species-specific responses. For instance, male *Betta splendens* exposed to 0.5–5 μg/L fluoxetine for three weeks showed less bold behaviour compared to unexposed fish (Dzieweczynski et al., 2016). Likewise, wild guppies (*Poecilia reticulata*) exposed for 28 days to 16 ng/L fluoxetine showed more freezing behaviour after a simulated predator attack and spent more time under plant cover than unexposed fish, suggesting increased anxiety (Saaristo et al., 2017). The fluoxetine-induced increase in anxiety-related behaviour in *N. furzeri* as observed in the current study is consistent with these

findings. This is further corroborated by an earlier reported increase in freezing behaviour upon a simulated antipredator attack, and a trend for spending less time in the centre of an open field upon a three-week exposure to 0.5–5 μg/L fluoxetine in *N. furzeri* (Thoré et al., 2018b). It should be noted that increased anxiety may also result in increased erratic swimming bouts (Mcdonald, 2017; Nowicki et al., 2014). Therefore, the observed fluoxetine-induced increase in maximum swimming acceleration, despite an overall decrease in activity level and swimming velocity, might further suggest a higher level of anxiety upon fluoxetine exposure.

While the observed decrease in activity and swimming velocity in response to fluoxetine exposure suggests an elevated level of anxiety, also increased lethargy may underlie this observation (Henry and Black, 2008; Mcdonald, 2017). Similar results are reported in literature, including a lower activity level in guppies upon exposure to 16 ng/L fluoxetine for 28 days (Saaristo et al., 2017) and in medaka (*Oryzias latipes*) upon exposure to 100 μg/L fluoxetine for 10 days (Ansai et al., 2016). These findings are, however, in contrast to what would be predicted by serotonin neurophysiology and the role of serotonin receptors, because serotonin is important for the generation rather than inhibition of locomotor activity (Mcdonald, 2017). That being said, SSRI's can affect different brain regions that are intricately involved in a variety of physiological and behavioural processes (Weinberger II and Klaper, 2014). Furthermore, fluoxetine is one of the least selective SSRI's on the market and may not only increase extracellular levels of serotonin but also trigger the release of other neurochemicals including norepinephrine and dopamine (Bymaster et al., 2002; Kellner et al., 2015). While the pharmacokinetics and -dynamics of SSRI's have been extensively studied in mammals, including humans, the exact mechanisms that underlie SSRI-induced behavioural effects in fish remain overall poorly understood (Eisenreich and Szalda-Petree, 2015; Mcdonald, 2017). Therefore, further studies are needed on the responses of wildlife to neurochemical exposure. Noteworthy, while a three-week exposure to 5 μg/L fluoxetine did not affect *N. furzeri* activity level in a previous study (Thoré et al., 2018b), these new results show that exposure to a much lower concentration (28 ng/L) over a longer period (74 days) does affect activity. This shows the need to assess effects of long-term exposure in environmental risk assessment of neurochemicals.

4.2. Fluoxetine disrupts daily activity patterns and the degree of impact depends on the time of the day

Although generally not considered, time of day was an important mediator of the behavioural impact of fluoxetine. This finding was anticipated given that, in humans, neurotransmitters such as serotonin are involved in circadian rhythmicity (Portas et al., 2000). In fish, the existence of daily rhythms in the HPI axis has been described in many species, predominantly with regard to fluctuating cortisol levels, and serotonin likely plays a regulatory role in fish circadian rhythmicity and associated behaviour (Cowan et al., 2017; Kreke and Dietrich, 2008). In addition, serotonin is involved in the production of melatonin, the time-keeping hormone, the rhythmic production of which enables the synchronisation of several physiological and behavioural processes with variation in environmental conditions (Cowan et al., 2017; Prokkola and Nikinmaa, 2018).

Most studies to date on daily rhythms in chemical effects are conducted on humans and other mammals (Prokkola and Nikinmaa, 2018), while only a handful of studies have explored such effects in aquatic organisms (Melvin, 2017; Melvin et al., 2016). Consistent with the current finding that fluoxetine disrupts daily activity patterns in *N. furzeri*, Melvin et al. (2016) found that wastewater treatment plant effluent abolished daily activity patterns in male mosquitofish (*Gambusia holbrooki*) after short-term (96 h) exposure, and showed that this finding was likely related to the presence of pharmaceutical- and personal care product-mixtures. However, a later study could not replicate this finding when male mosquitofish were exposed to 100 μg/L fluoxetine over a period of one week (Melvin, 2017). Next to potential species- and concentration-dependent responses, it is reasonable to expect that also differences in exposure duration explain why fluoxetine treatment affected daily activity patterns in the current study. To our knowledge, this is the first study to test daily rhythms with relation to chemical effects at low, environmentally-relevant levels and over longer, realistic timeframes. The results of this study are therefore an essential step to relate daily rhythms to toxicity outcomes in wildlife and better estimate the environmental impact of neurochemicals.

4.3. Why behavioural rhythms should not be overlooked in eco(toxico)logy

While the possible ecological consequences of altered activity and anxiety-like behaviour have been discussed extensively in literature, the impact of changes in daily activity patterns has received far less attention. Such rhythms serve to tune animal behaviour to environmental conditions, and are likely under strong selection as they may be key to several fundamental ecological interactions (Bulla et al., 2016; Westwood et al., 2019). For instance, biological rhythms allow individuals to temporally segregate their daily activities to facilitate the co-existence of competitors and predator avoidance (Alanärä et al., 2001; Kronfeld-Schor and Dayan, 2003). Biological rhythms also allow for synchronizing daily activities, including reproductive behaviours, group foraging and communal defence (Bulla et al., 2016). Although such rhythms may have high adaptive value and affect the long-term viability of populations (Prokkola and Nikinmaa, 2018), so far they were predominantly studied to elucidate their mechanistic underpinnings (Westwood et al., 2019). In contrast, they were only recently integrated in an ecological and evolutionary framework (Bulla et al., 2016; Thoré et al., 2019; Westwood et al., 2019). Because chemical-induced changes in biological rhythms may carry severe fitness consequences, considering recurrent daily behavioural variation in ecotoxicology may be crucial to fully understand the ecological consequences of such changes. This is especially important given that the presence of neurochemicals in the environment is expected to increase due to their increasing and continuous use. Next to fluoxetine also other SSRI's such as citalopram and fluvoxamine pollute the environment and, combined, have been detected at concentrations of up to 800 μg/L (Martin et al., 2019b). Moreover, not only neuroactive pharmaceuticals increasingly pollute the environment, but also other neurochemicals such as neonicotinoid pesticides (Wood and Goulson, 2017).

In the current study, only two time points were considered (i.e., morning vs. evening) to show that important variation may be ignored if data is collected at a single time point. Ideally, however, day-round (24 h) behavioural patterns should be assessed to explore the relationship between daily behavioural changes and the effects of pollution. Furthermore, it is important to note that animals may not only display behavioural rhythms over short (e.g., daily variation) but also over longer temporal scales, including seasonal variation. This is especially relevant to consider in the context of long-term exposure studies with species that show circannual rhythms, but has so far escaped the attention of most ecotoxicological studies (Prokkola and Nikinmaa, 2018). Likely, daily and seasonal rhythms are at least partly governed by common

biological time-keeping mechanisms (Helm et al., 2013), suggesting that in an ecotoxicological framework they should ideally be studied together.

5. Conclusions

Neurochemicals increasingly pollute the environment and affect non-target organisms. Although many studies document behavioural effects of (neuro)chemicals in animals, natural short-term variability in behaviour is largely overlooked as a potential mediator and sensitive endpoint of chemical impact. This is the first study to test daily behavioural variation in fish with relation to chemicals effects at low, environmentally-relevant levels over a realistic timeframe. We show that chronic exposure to 28 ng/L fluoxetine lifts daily activity patterns and drives an increase in anxiety-like behaviour that is slightly more pronounced in the evening compared to the morning. These findings suggest that natural biological rhythms may confound test results and call for their integration in ecotoxicological studies.

Author statement

Eli S.J. Thoré: Conceptualization, Methodology, Formal analysis, Writing - original draft. Luc Brendonck: Supervision. Tom Pinceel: Writing - original draft.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116738.

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