Science of the Total Environment 917 (2024) 170405

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Sub-chronic exposure to paroxetine disrupts ecologically relevant behaviours in fish

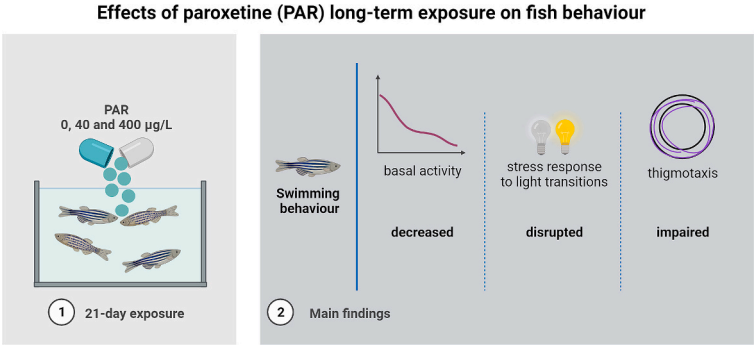
Carla S.S. Ferreira [a,*], Cátia Venâncio [a], Mónica Almeida [a], Isabel Lopes [a], Peter Kille [b], Miguel Oliveira [a]

[a] Centre for Marine and Environmental Studies (CESAM), Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
[b] School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK

HIGHLIGHTS

- Paroxetine (PAR) effects on zebrafish behaviour were studied under light and dark conditions.
- PAR exposed fish displayed reduced swimming activity and disrupted response to light transitions.
- The applied integrative approach proved to be sensitive for screening PAR behavioural effects.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Julian Blasco

Keywords:
Selective serotonin reuptake inhibitors
Second-generation antidepressants
Zebrafish
Sub-chronic effects
Behaviour modification

ABSTRACT

The functional conservation of important selective serotonin reuptake inhibitor (SSRI) targets in non-target organisms raises concerns about their potential adverse effects on the ecosystems. Although the environmental levels of SSRIs like paroxetine (PAR) have risen, the knowledge regarding the effects of long-term exposure to PAR is limited. This study investigated the impact of sub-chronic exposure (21 days) to two sub-lethal concentrations of PAR (40 and 400 µg/L) on the behaviour of adult zebrafish in different scenarios: basal activity (under dark and light conditions), stress response (evoked by sudden light transitions) and stress response recovery. A new framework was employed for the integrative study of fish's swimming performance based on their innate ability to respond to light shifts. Several swimming-associated parameters (e.g., total swimming distance, time of inactivity, swimming angles) and thigmotaxis were monitored for an integrated analysis in each scenario. Data revealed reduced swimming activity, impaired behavioural response to stress and alterations in stress recovery of PAR-exposed fish. An anxiolytic effect was particularly noticeable in fish basal swimming activity in the dark at 400 µg/L and in the behavioural response to stress (from dark to light) and stress recovery (from light to dark) for organisms exposed to 40 µg/L.

The detected PAR-induced behavioural modifications suggest a disruption of brain glucocorticoid signalling that may have implications at the individual level (e.g., changing behavioural responses to predators), with

* Corresponding author at: Centre for Marine and Environmental Studies (CESAM), Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal.
E-mail address: csofia@ua.pt (C.S.S. Ferreira).

https://doi.org/10.1016/j.scitotenv.2024.170405
Received 27 September 2023; Received in revised form 8 January 2024; Accepted 22 January 2024
Available online 26 January 2024

potential repercussions on the population and community levels. Therefore, the applied protocol proved sensitive in detecting behavioural changes induced by PAR.

1. Introduction

The consumption of selective serotonin reuptake inhibitors (SSRI) antidepressants, like paroxetine (PAR), increased significantly due to the COVID-19 pandemic associated with a higher incidence of depression and anxiety (Diaz-Camal et al., 2022; Ferreira et al., 2023a). Hence, their environmental presence is expected to increase as conventional wastewater treatment plants (WWTPs) have shown to be ineffective in removing them, constituting a global challenge to the aquatic environment (Hossain et al., 2021). Previous studies reported PAR concentrations in surface waters of up to 270 ng/L (Arnnok et al., 2017; Sumpter and Margiotta-Casaluci, 2022) and up to 3380 ng/L in WWTP effluents from pharmaceutical manufacturing industries (Kleywegt et al., 2019), with untreated sewage showing even higher levels, reaching up to 39.73 μg/L (Salgado et al., 2011). Because untreated sewage is occasionally directly released into surface waters, the former can significantly impact the latter (Nowakowska et al., 2020).

Current data show that SSRIs can affect the behaviour of non-target organisms (Ansai et al., 2019; de Farias et al., 2020; Huang et al., 2019; Martin et al., 2017, 2020; Nowakowska et al., 2020; Pelli and Connaughton, 2015; Saaristo et al., 2017; Salahinejad et al., 2022; Venkatachalam et al., 2023). For instance, some studies addressing the effects of short-term exposure to PAR (1–400 μg/L) in zebrafish embryos/larvae (3–6 days post-fertilization) reported early hatching, developmental abnormalities (scoliosis), and altered swimming activity (Nowakowska et al., 2020; Huang et al., 2019; Ferreira et al., 2023b). Disruption of zebrafish juveniles´ swimming activity and stress response has been reported after 120 h of exposure to a commercial formulation of PAR (40 and 400 μg/L) (Ferreira et al., 2023b). Moreover, decreased anxiety levels and fertility in zebrafish (juveniles and adults) were also reported after 35 and 135 days of exposure to 100 μg/L PAR (Venkatachalam et al., 2023).

Nonetheless, the available data concerning the effects of PAR on fish behaviour is scarce, particularly regarding medium and long-term effects under environmentally realistic scenarios (Ferreira et al., 2023a). The limited studies addressing the impact of PAR in fish did not consider its influence under different light conditions, a handicap as fish behave differently under light and dark conditions due to an intrinsic circadian clock that allows them to detect changes in light conditions. Sudden light transitions have been reported as stress inducers in fish that associate these alterations as potential threats (Ferreira et al., 2023b, 2023c). They appear as a valuable approach to studying the impact of environmental contaminants on stress and stress recovery responses. For instance, fish seem to perceive the light/dark transition as the shadow of a predator (Ferreira et al., 2023b). Since fish tend to quickly return to their pre-stress swimming activity levels, any contaminant-induced changes in stress-related behaviours may directly interfere with fish's ability to adjust and optimize their response (e.g., predator escape/avoidance) to a perceived threat (Ferreira et al., 2023b, 2023c). The analysis of such behavioural modifications can be useful in providing valuable insights into the potential impact on individual fitness (e.g., ability to respond to additional stressors).

The assessment of basal swimming performance in both light and dark environments also holds significant environmental relevance. Any changes in fish's usual swimming behaviour, beyond directly impacting individual fitness (e.g., feeding capabilities and nutritional status), may also influence dispersal, migration, and reproductive activities, vital for population survival (Bertram et al., 2022; Brodin et al., 2014; Faimali et al., 2017; Ferreira et al., 2023c).

Numerous studies have used zebrafish (*Danio rerio*) as a model organism to explore the effects of SSRIs on fish. These investigations offer valuable insights applicable to vertebrates, including humans, due to the high genomic and disease ortholog similarities along with the significant conservation of monoaminergic systems (Adhish and Manjubala, 2023; Xie et al., 2022). This study was motivated by the absence of data on prolonged PAR exposure on fish swimming activity and behavioural response to stress under different light conditions. Thus, for this purpose, the effects of sub-chronic exposure to PAR were studied on *D. rerio* behaviour in three different scenarios: basal activity, stress response and stress recovery, based on fish's innate ability to react to light changes. Two concentrations were tested: 40 and 400 μg/L, selected according to previous tests conducted by the team (Ferreira et al., 2023b). An integrated approach was employed to assess fish behaviour in each one of the different scenarios, incorporating different swimming-related parameters (total swimming distance, total time of inactivity and swimming angles) and thigmotaxis measurement to evaluate potential disruption of the normal swimming pattern and stress response induced by PAR.

2. Material and methods

2.1. Zebrafish husbandry

The zebrafish used in the experimental assay were obtained through crossbreeding of animals from the Department of Biology, University of Aveiro (Portugal) maintained under controlled conditions, as described by Ferreira et al. (2023b). Animals were reared until the adult stage (8 months old) and fed with a commercially available artificial diet (GEMMA Micro 500, Skretting USA).

2.2. Zebrafish exposure

Zebrafish adults (8 months old) measuring 2.78 ± 0.25 cm and displaying no apparent indicators of compromised health (i.e. presenting normal morphology and behaviour) were exposed for 21 days to 0, 40, and 400 μg/L of PAR (paroxetine hydrochloride hemihydrate (CAS 110429–35-1; TCI Europe). A stock solution of PAR (40 mg/L) was prepared by dissolving the powder in reconstituted water [Milli-Q water with the addition of marine salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) to adjust conductivity to 800 ± 50 μS/cm] and further diluted to the desired test solutions. For this purpose, zebrafish males and females were randomly distributed into 3 experimental groups (with 9 replicates per treatment) at a density of 4 fish (2 females and 2 males) per 0.75 L of test media: control (without PAR), 40 PAR (40 μg/L of PAR), and 400 PAR (400 μg/L of PAR). Animals were daily fed with GEMMA Micro 500 (Skretting USA) and checked for mortality and any signs of distress. Every other day, 70 % of the test medium was renewed. No mortality was observed throughout the exposure period.

The experimental procedures complied with the 3 R's policy and with legal and ethical standards outlined in the Portuguese legislation (Decreto-Lei 113/2013) and European laws (Directive 2010/63/EU) concerning the welfare of animals employed for scientific research and were approved by the Animal Ethics Committee of the University of Aveiro, Portugal. The experiments were carried out by researchers who possessed FELASA certification.

2.3. Assessment of swimming performance

After the 21-day exposure period, the behaviour of the animals was recorded using the Zebrabox tracking system (Viewpoint, Lyon, France) that allowed the tracking of the movement of each fish. The behavioural trials were conducted individually in a rectangular plastic tank (9.4 cm

wide and 14.1 cm long) with 2.5 cm of the test media. The tracking process lasted for 16 min and was preceded by an initial acclimation period of 10 min in the dark. The tracking session started with 3 min of darkness, followed by 10 min of light, and finished with an additional 3 min in the dark (Fig. 1).

2.3.1. Basal swimming activity

The basal swimming performance, under dark and light conditions, was assessed as described by Ferreira et al. (2023c) (Fig. 1). The fish's movement patterns were analysed considering the displayed swimming angles. In this analysis, eight classes of angles were established and analysed as described by Zhang et al. (2017) and Almeida et al. (2019). These classes distinguish between high-amplitude angles (1 and 8; 2 and 7) that represent zig-zag movements with pronounced changes in direction (indicating erratic or stressed behaviour), medium-amplitude angles (3 and 6) corresponding to average turns, and low-amplitude angles (4 and 5) signifying straightforward movements. Thigmotaxis measurement was also monitored to assess anxiety levels based on the innate preference of zebrafish to swim near the periphery of the test area, avoiding the centre of the open area. Additionally, the total swimming distance and the total inactivity time were also evaluated to provide a more comprehensive evaluation of the fish's swimming behaviour.

2.3.2. Behavioural stress response and stress recovery

To assess the possible effect of PAR on stress response and light sensitivity, behavioural modifications induced by sudden light transitions, both from dark to light and from light to dark, were evaluated, considering total swimming distance, total time of inactivity, thigmotaxis and swimming angles. These parameters were evaluated at two-time points: 1 and 3 min immediately before and after the light condition transition, as described by Ferreira et al. (2023c) (Fig. 1). This approach allowed the study of the immediate stress response induced by the light variations, as well as the ability of fish to recover from this stress stimulus.

2.4. Data analysis

For the analysis of behavioural endpoints, a one-way analysis of variance (ANOVA) was conducted when the assumptions of normality and homoscedasticity of variances were fulfilled. Subsequently, the pairwise post-hoc Holm-Sidak method (using SigmaPlot V.14.0 software) was employed to assess the significant differences between the experimental groups. If the assumptions were not met, a non-parametric ANOVA was used followed by the multicomparison Dunn's test. To evaluate differences in behaviour between dark and light conditions, a Student *t*-test was employed. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Basal swimming activity

The *D. rerio* exposure to the two tested PAR concentrations led to significant changes in the fish's swimming performance and swimming patterns, under both light and dark periods (Fig. 2A and B). Regarding locomotor activity, an overall similar pattern of response was found for light and dark conditions with 40 and 400 μg/L PAR-exposed fish swimming significantly less than the control (Fig. 2A). PAR-exposed fish displayed no significant differences between light conditions in terms of distance moved, unlike controls that displayed a higher total swimming distance in the light (Fig. 2A).

The total time spent in inactivity in dark and light conditions displayed an opposite pattern of response. Under dark conditions, the time of inactivity increased with PAR concentration increase (Fig. 2B), whereas under light conditions, fish total inactivity time in exposed fish was significantly lower than in the control (Fig. 2B). All pairwise comparisons between dark and light conditions within each PAR treatment revealed significant differences, with control fish, in light, being more inactive than PAR-exposed fish (Fig. 2B). Under dark conditions, fish exposed to 400 μg/L PAR displayed decreased thigmotaxis when compared to control and 40 μg/L PAR-exposed fish (Fig. 2C) whereas in light conditions no significant differences between experimental groups were found in terms of thigmotactic behaviour (Fig. 2C).

The fish trajectory measured through the analysis of swimming angles has shown that under light conditions exposed fish (40 and 400 μg/L) exhibited decreased frequency of high amplitude angles of class 1 and 8 (Fig. 2D) compared to control. In the dark condition, no significant differences were found between exposed and non-exposed organisms for this class of angles (Fig. 2D). The effects of PAR exposure on fish trajectory were further noticeable on 400 μg/L PAR exposed fish that also displayed decreased frequency of class 2 and 7 angles in both light and dark conditions in comparison with control and 40 μg/L fish (Fig. 2E). No differences between experimental groups were observed in terms of medium and low amplitude angles (Fig. 2F and G).

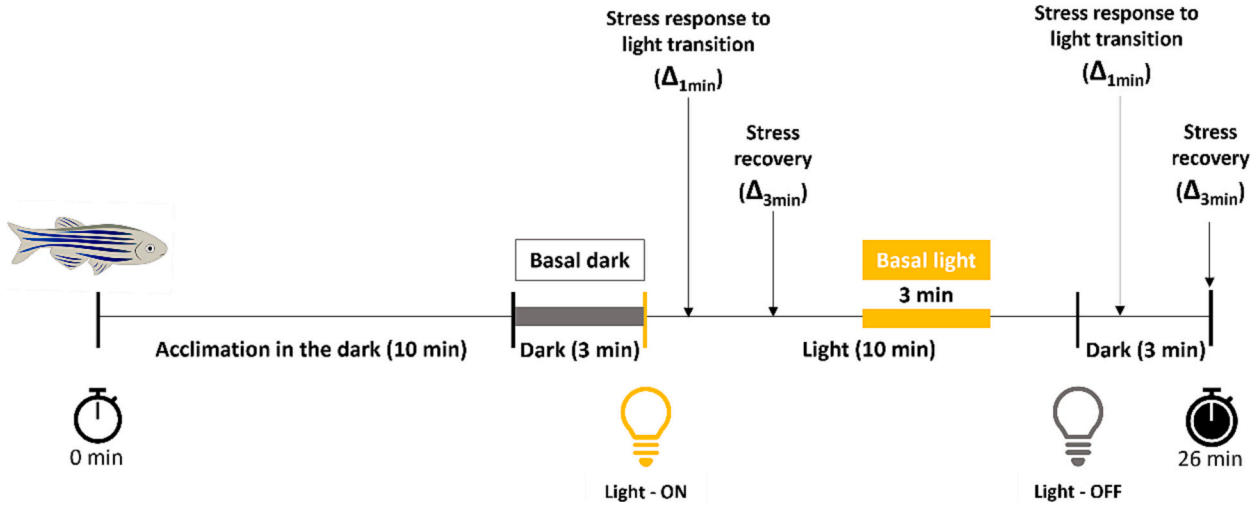


Fig. 1. Schematic representation of the behavioural assessment design with the different time points used to analyse fish basal swimming activity (in dark and light conditions) and the response and recovery from stress induced by sudden light transitions (from dark to light and from light to dark).

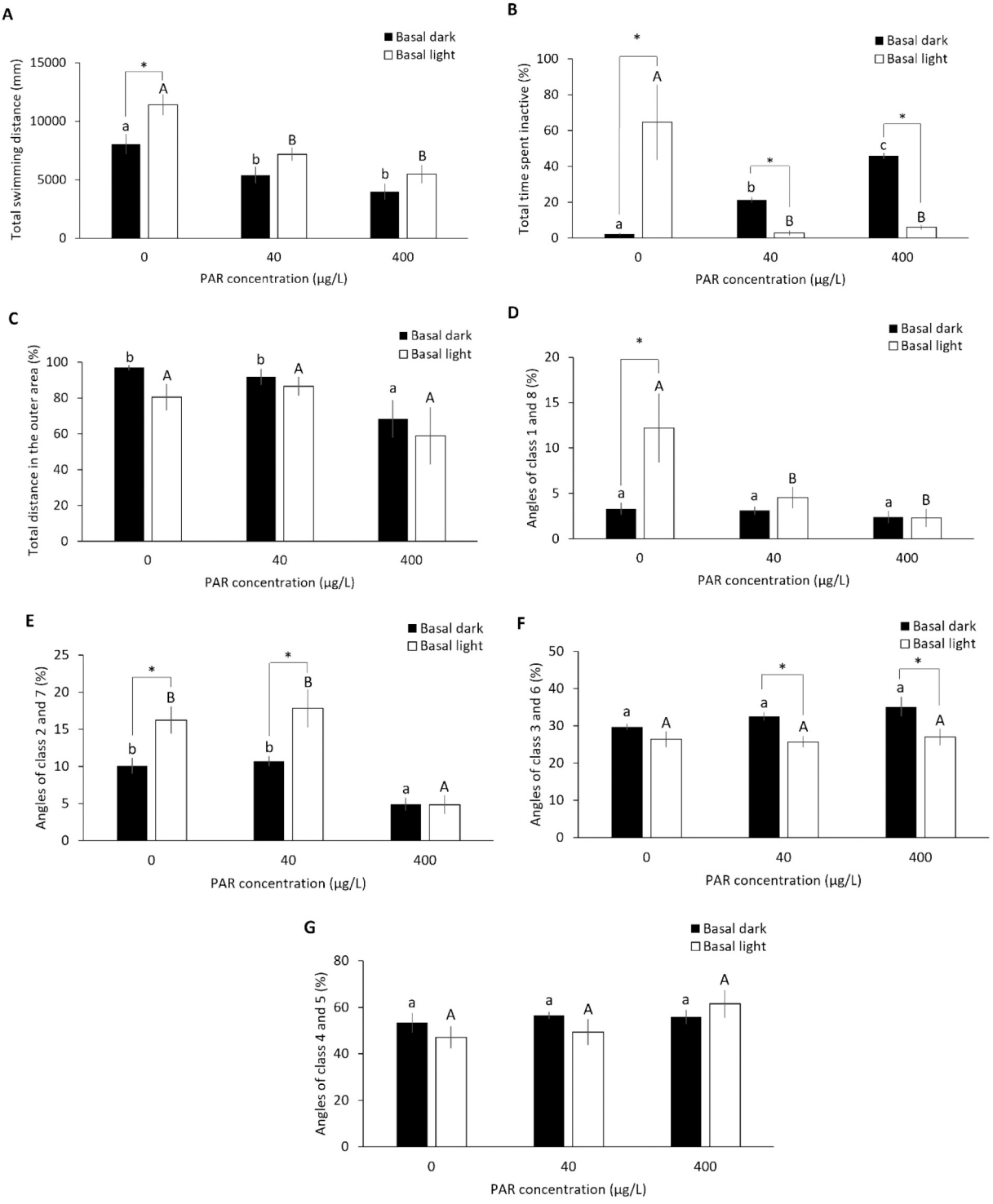


Fig. 2. Paroxetine (PAR) sub-chronic (21 days) effects on *Danio rerio* adults' basal swimming activity under light and dark conditions. The parameters analysed were: A) Total Swimming Distance (mm); B) Total Time Spent in Inactivity (%); C) Total Distance in the Outer Area (%); D) Angles of Classes 1 and 8 (%); E) Angles of Classes 2 and 7 (%); F) Angles of Classes 3 and 6 (%); G) Angles of Classes 4 and 5 (%). The dark period is represented by black bars, and the light period is represented by white bars. Values are presented as mean ± standard error. * indicates significant differences between the dark and light periods for each tested condition. Different lower-case letters indicate significant differences across treatments for dark conditions and different capital letters for light conditions. Statistical analysis was performed using a one-way ANOVA followed by Holm-Sidak's test and an ANOVA on ranks followed by Dunn's test ($p < 0.05$).

3.2. Behavioural stress response evoked by sudden light transitions and recovery from stress

3.2.1. The dark/light transition

The total swimming distance and thigmotaxis measured during the stress response to the dark/light transition were significantly lower in 400 µg/L PAR exposed organisms than in control and 40 µg/L PAR (Fig. 3A and C). In response to the same light stressor, 40 µg/L PAR exposed fish also revealed decreased swimming distance as well as increased time of inactivity when compared to the control and 400 µg/L

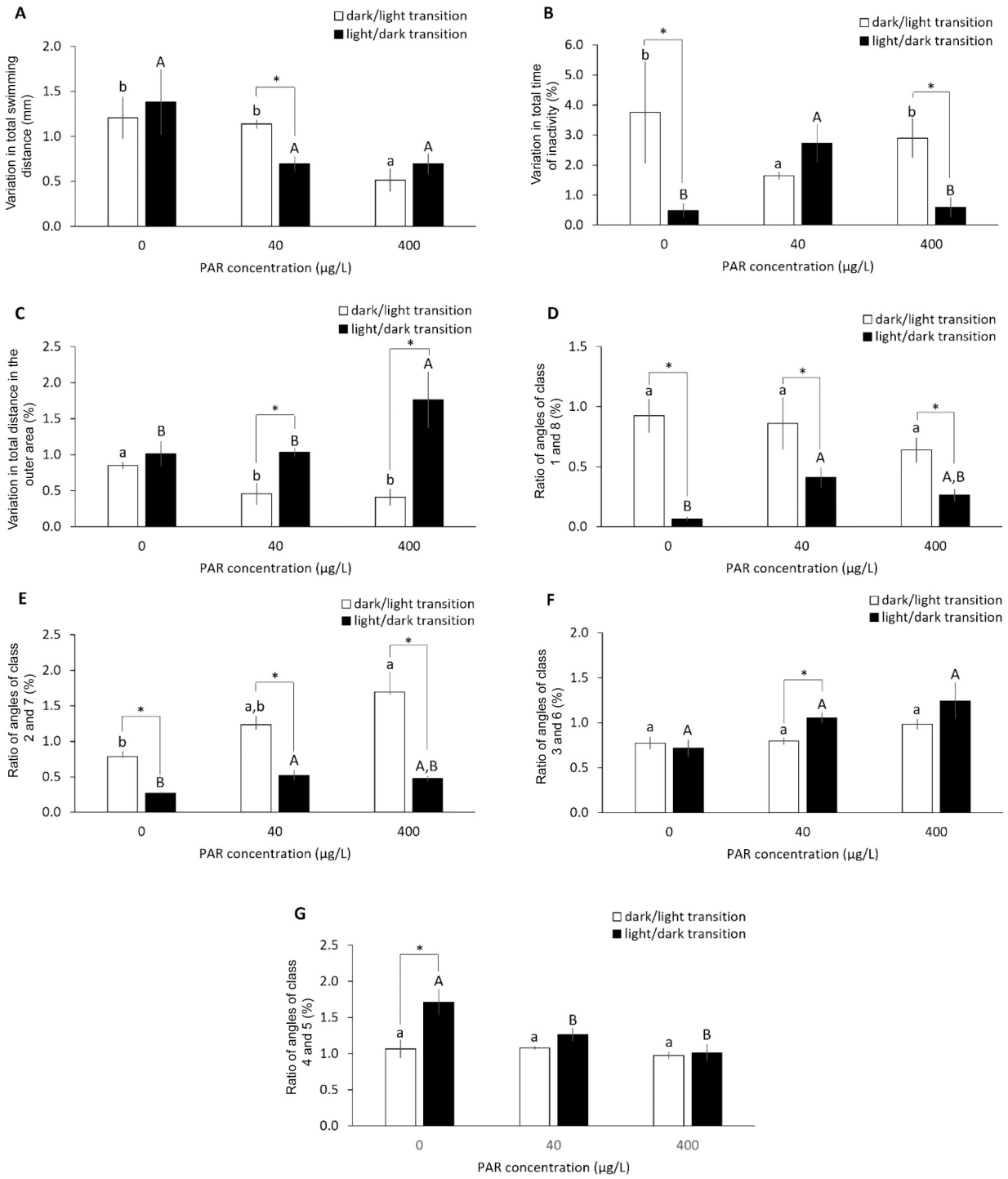


Fig. 3. Behavioural response to stress induced by sudden light transitions (from dark to light and from light to dark) of *Danio rerio* adults after paroxetine (PAR) sub-chronic (21 days) exposure. The parameters analysed were: A) Total Swimming Distance (mm); B) Total Time Spent in Inactivity (%); C) Total Distance Outer (%); D) Angles of Classes 1 and 8 (%); E) Angles of Classes 2 and 7 (%); F) Angles of Classes 3 and 6 (%); G) Angles of Classes 4 and 5 (%). Values are presented as mean ± standard error. The dark/light transition is represented by white bars, and the light/dark transition is represented by black bars. * indicates significant differences between the dark/light and the light/dark transitions for each tested condition. Different lower-case letters indicate significant differences across treatments for dark conditions and different capital letters for light conditions. Statistical analysis was performed using a one-way ANOVA followed Holm-Sidak's test and an ANOVA on ranks followed by Dunn's test ($p < 0.05$).

PAR exposed fish (Fig. 3B). In terms of swimming angles, no significant differences between treatments were found concerning high amplitude (class 1 and 8), medium (class 3 and 6) and low (class 4 and 5) amplitude angles (Fig. 3D, F and G). However, significant differences were found in the frequency of class 2 and 7 angles (high amplitude), with fish exposed to 400 µg/L PAR displaying higher frequency than control and 40 µg/L (Fig. 3E).

Regarding stress recovery, no statistical differences were found between experimental conditions concerning total swimming distance and distance spent in the outer zone of the well (Fig. 4A and C). However, fish exposed to PAR 40 µg/L revealed a longer period of inactivity than control and 400 µg/L PAR fish (Fig. 4B). Effects of PAR exposure were

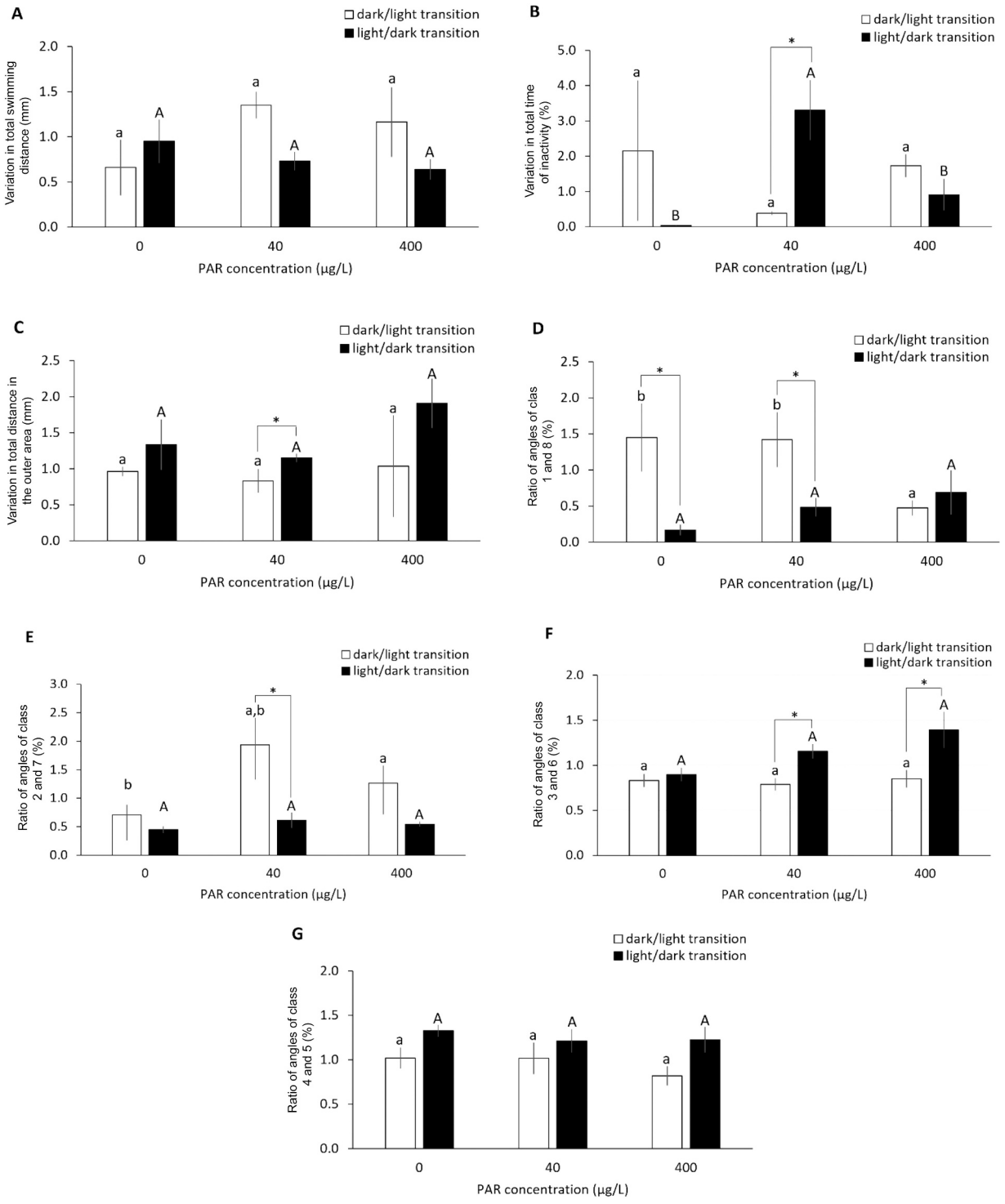


Fig. 4. Recovery response of *Danio rerio* adults to stress induced by sudden light transitions (from dark to light and from light to dark) after paroxetine (PAR) sub-chronic (21 days) exposure. The parameters analysed were as follows: A) Total Swimming Distance (mm); B) Total Time Spent in Inactivity (%); C) Total Distance Outer (%); D) Angles of Classes 1 and 8 (%); E) Angles of Classes 2 and 7 (%); F) Angles of Classes 3 and 6 (%); G) Angles of Classes 4 and 5 (%). Values are presented as mean ± standard error. The dark/light transition is represented by white bars, and the light/dark switch is represented by black bars. * indicates significant differences between the dark/light and the light/dark transitions for each tested condition. Different lower-case letters indicate significant differences across treatments for dark conditions and different capital letters for light conditions. Statistical analysis was performed using a one-way ANOVA followed by Holm-Sidak's test and an ANOVA on ranks followed by Dunn's test ($p < 0.05$).

also noticeable in terms of class 2 and 7 swimming angles (high amplitude), with 400 µg/L PAR exposed fish displaying lower frequency of this class of angles than control (Fig. 4E). These organisms (400 µg/L) also showed a lower frequency of high amplitude angles of class 1 and 8, unlike control and 40 µg/L exposed fish (Fig. 4D). In terms of medium and low amplitude angles, no effects of PAR were observed in these classes of angles (Fig. 4F and G).

3.2.2. *The light/dark transition*

The analysis of the stress response to the light/dark shift revealed a

non-significant decrease trend in the total distance travelled by fish exposed to PAR when compared to the control (Fig. 3A). Fish exposed to 40 μg/L PAR reacted to this light stress increasing inactivity time (unlike control and 400 μg/L) (Fig. 3B), whereas those exposed to 400 μg/L PAR increased thigmotaxis (when compared to control and 40 μg/L PAR-exposed fish) (Fig. 3C). Fish exposed to PAR 40 μg/L also exhibited heightened frequency of high amplitude angles of class 1, 2, 7 and 8 in comparison to non-exposed fish (control) (Fig. D and E). No differences between exposed and non-exposed organisms were found in terms of medium angles (class 3 and 6), but significant differences were found in low amplitude angles (class 4 and 5) with exposed fish presenting decreased frequency of this class of angles (Fig. 3F and G).

Considering the recovery response from stress induced by this light transition, no significant differences between PAR treatments and control were found in swimming distance and distance swam in the outer zone (Fig. 4A and C). However, the same U-inverted response, previously observed in the stress response, was found for total time spent in inactivity, with 40 μg/L PAR-exposed fish being significantly less active than control and 400 μg/L PAR-exposed fish (Fig. 4B). No significant differences in fish trajectory angles were found between control and PAR treatments (Fig. 4D, E, F and G).

3.2.3. Comparison of fish behavioural response pattern between both dark/light and light/dark transitions

When comparing the behavioural response to stress induced by each light transition, a similar pattern of response was found for control and 400 μg/L PAR-exposed fish that swam a longer distance following the light/dark transition than after the dark/light switch (Fig. 3A). However, fish exposed to 40 μg/L revealed an opposite trend swimming longer distances after the dark/light transition. Major differences were also found for total time spent in inactivity, where a U-shaped response was found in dark/light transition, whilst an inverted U-shaped response in light/dark transition (Fig. 3B), with 40 μg/L PAR presenting distinct response patterns between both light transitions (dark/light versus light/dark). Fish exposed to 40 μg/L PAR did not display a differentiated response concerning inactivity time after being subjected to the two types of light stressors. Concerning the total distance covered by fish in the outer area (thigmotactic behaviour), both control and PAR-exposed fish revealed a similar trend to swim higher distances upon the light/dark switch (Fig. 3C). In 40 μg/L PAR-exposed fish significant differences between light transitions were found, unlike the other experimental groups (Fig. 3C).

In terms of swimming trajectory, fish from all experimental groups exhibited a higher proportion of high amplitude angles (class 1, 2, 7 and 8) in response to the dark/light transition (Fig. 3D and E). However, unlike control fish, 40 and 400 μg/L PAR-exposed fish did not respond differently to light transitions in terms of class 4 and 5 angles. (Fig. 4G).

Concerning the recovery response, fish did not respond differently to light transitions in terms of total swimming distance and frequency of class 4 and 5 angles (low amplitude), regardless of the experimental group (Fig. 4A and G). However, regarding inactivity time, total distance swam in the outer zone of the arena, and class 2 and 7 angles frequency, fish exposed to 40 μg/L PAR displayed a differentiated response to the two light transitions unlike control and 400 μg/L PAR fish (Fig. 4B, C and E). Concerning the frequency of class 3 and 6 angles (medium amplitude), PAR-exposed fish (40 and 400 μg/L PAR) reacted differently to each light transition unlike the control (Fig. 4F). Additionally, fish exposed to 400 μg/L PAR also displayed disruption of the normal response pattern in the frequency of high amplitude angles (class 1 and 8) as these organisms did not respond differently to the light transitions throughout the recovery response, unlike the other experimental groups (Fig. 4D).

4. Discussion and conclusions

The use of psychoactive therapeutic agents became a recurrent and growing trend, with unforeseen consequences for the environment. In the past years, their environmental presence has raised low levels of concern, as no mortality has been reported at environmental levels. Nonetheless, they are pharmacologically active biomolecules and their potential to adversely affect biota in the long term and on ecologically relevant endpoints, such as behaviour is far from being understood. This study was motivated by the critical imperative to address a knowledge gap seeking to understand the medium-term effects of sub-lethal PAR levels on behavioural endpoints in environmentally relevant scenarios that may provide relevant information concerning biological and ecological implications that remain unexplored. As far as the authors are aware, data are absent regarding the enduring impacts of PAR on fish basal swimming activity and behavioural response to stress under different light conditions.

Swimming behaviour is directly correlated with several fitness-related behaviours in many aquatic organisms (Faimali et al., 2017; Ferreira et al., 2023c). Specifically for fish, swimming performance has a direct connection to a wide spectrum of ecologically relevant behaviours, playing an important role in encounter rates, predator-prey interactions (to eat or to be eaten), and a key element in dispersal and migration-related behaviours (Brodin et al., 2014; Faimali et al., 2017; Ferreira et al., 2023c). It is also directly associated with anxiety and fear responses that are part of a repertoire of coping strategies to optimize and enhance organisms' fitness in response to any potential threats (e.g., predation) (Salahinejad et al., 2022). In gregarious fish species, any disturbance to swimming performance can also jeopardize the cohesion of shoaling behaviour, consequently heightening vulnerability to predation (Brodin et al., 2014; Faimali et al., 2017; Ferreira et al., 2023b). This study data revealed that the anxiolytic effect induced by PAR was more evident under dark conditions, where exposed fish exhibited reduced swimming distance and inactive periods. These effects were particularly pronounced in fish exposed to 400 μg/L, as decreased erratic behaviour and thigmotaxis reduction were exclusive to these organisms. Under light conditions, exposed fish also showed reduced swimming distance and erratic behaviour, supporting decreased stress behaviour. The effects of PAR were more evident under dark conditions a period where fish displayed a higher number of swimming-associated parameters affected, supporting the idea that fish behavioural response is dependent on the type of light stimulus, probably due to the involvement of a different network of glucocorticoid-mediated signalling pathways. In a study addressing the effects of zebrafish juveniles' acute (96 h) exposure to PAR (40 and 400 μg/L), Ferreira et al. (2023b) also observed an anxiolytic effect. Although, in this case, it was more evident in fish exposed to 40 μg/L under light conditions. These different outcomes are probably related to the exposure period and with fish life stage. In the context of human physiology, the therapeutic effects of SSRIs on individuals only become noticeable after a treatment period spanning 3–4 weeks. The pharmacokinetics and pharmacodynamics of SSRIs are complex and not well understood, particularly concerning long-term exposures. In addition, there may be potential interference from sex-related hormones, since in the juvenile stage, unlike the adult stage, the fish have not yet reached sexual maturity. The assessment of behavioural changes resulting from sudden light transitions revealed that exposed and non-exposed fish responded differently to this stress stimulus. The data from this study offers evidence that prolonged exposure to PAR can influence both the initial stress response and the subsequent recovery ability of fish from stressors. A study performed by Ferreira et al. (2023b), found that the sudden transition from dark to light acts as a startling trigger resulting in fish increased stress. In this study, in response to the dark/light transition, control fish exhibited increased freezing (supported by a heightened inactivity period) and erratic swimming. These observations are consistent with a fright reaction to a sudden scare, suggesting heightened stress. In this study, upon this light transition, PAR-exposed fish (40 μg/L and 400 μg/L), displayed lower thigmotaxis than control fish and concentration-specific responses in erratic behaviour (increased only at 400 μg/L) and in

swimming distance (decreased only at 40 μg/L). These PAR-induced changes in the stress response suggest a heightened reaction in organisms exposed to 400 μg/L, while exposure to 40 μg/L appeared to have an anxiolytic effect. The effects of PAR on fish's ability to recover from this stressor were observed in animals exposed to 400 μg/L which revealed a higher frequency of high amplitude angles (class 2 and 7) than the control group, which can be associated with increased stress-related behaviour. These outcomes highlight the relevance of light transitions as a good model for assessing potential anxiolytic effects.

Upon light to dark shift, control fish reacted with a decrease in freezing behaviour (as they spend less time inactive), an increase in swimming distance (although not statistically significant), and a tendency towards straight motion. Ferreira et al. (2023b), suggested that this light transition may be interpreted by fish as a potential threat, such as the shadow of a predator, to which they adjust their behaviour to minimize the risk of predation. Unlike control fish, PAR-exposed fish upon this light stimulus displayed reduced straight motion. Additionally, fish exposed to 40 μg/L PAR increased erratic behaviour, and 400 μg/L organisms exhibited increased thigmotaxis, suggesting increased stress/anxiety-like behaviour. However, while 400 μg/L PAR fish displayed no differences in the recovery to this light transition (light/dark), fish exposed to 40 μg/L appeared to have a delay in this recovery response supported by increased inactivity periods. These different response patterns to these two different light transitions (from dark to light and from light to dark) are intrinsically linked to fish's ability to perceive and be sensitive to light, thus responding differently depending on the type of stimulus. Overall, the present study data suggest that PAR may disturb light perception and subsequently impact the fish's optomotor response, a visually mediated behaviour. A possible pathway for the observed behavioural alterations may be related to the disruption of the functional connections between the retina and the hypothalamus-pituitary-interrenal (HPI) axis linking light perception to glucocorticoid production, thereby influencing stress response (Muto et al., 2013; Sakamoto and Sakamoto, 2019). Additionally, the existence of functional interactions between the serotonergic and the adrenergic system and the HPI axis, may also been involved in the observed PAR-induced behavioural changes, as it is known that SSRIs, like PAR, act on the serotonergic system, thus playing a significant role in modulating the stress response (Kreke and Dietrich, 2008; Sumpter and Margiotta-Casaluci, 2022).

PAR long-term anxiolytic like-effects have recently been reported in zebrafish juveniles (90- and 120-days post-fertilization) exposed for 12 weeks during development to PAR at 100 μg/L (Venkatachalam et al., 2023). Previous studies with the SSRI fluoxetine also described behavioural alterations (reduced feeding, exploratory and swimming behaviours) and anxiolytic responses in adults and juveniles of several fish species (e.g., *D. rerio*, *Oryzias latipes, Gambusia holbrooki*, *Poecilia reticulata*, *Pimephales promelas*) after chronic exposure to a fluoxetine concentration range from 0.004 up to 32100 μg/L (de Farias et al., 2020; Martin et al., 2017, 2020; Pelli and Connaughton, 2015; Saaristo et al., 2017). Nevertheless, these studies exclusively conducted the behavioural evaluation during daylight hours. Considering that fish possess the capacity to perceive changes in light and to react accordingly, it becomes crucial to understand how SSRIs may modulate behaviour under different light conditions (Ferreira et al., 2023b). The present study findings suggest a potential anxiolytic effect of prolonged exposure to PAR, in light and dark conditions (more pronounced under dark conditions and at 400 μg/L) and upon the sudden shift from dark to light for organisms exposed to 40 μg/L. In comparison, a heightened reaction was observed in response to the light/dark shift (more noticeable at 400 μg/L). Ferreira et al. (2023b) also reported an anxiolytic effect in the light and during recovery to the dark/light transition, and a higher stress behaviour upon the light/dark switch (more pronounced at 40 μg/L), in zebrafish juveniles after acute exposure (120h) to a commercial formulation of PAR (40 and 400 μg/L). The latest appeared to elicit more effects as a higher number of swimming-associated parameters were reported to be affected during basal activity and throughout stress response and stress recovery (Ferreira et al., 2023b). This fact may be due to the existence of long-term compensatory mechanisms, such as serotonin receptors' desensitization, associated with SSRIs chronic exposure (McDonald, 2017). In humans, prolonged SSRI treatment leads to a significant decrease in serotonin transporter (SERT) expression and function, despite increased extracellular serotonin levels (Homberg et al., 2007). In addition, exposed fish seem to become more reactive to stress induced by the light/dark shift but less sensitive to the dark/light switch. Further investigation is required to gain deeper insights into these different response patterns of PAR-exposed fish during each light transition, shedding light on the underlying factors responsible for these differences.

The observed effects of PAR on fish swimming behaviour and stress response may be translated into alterations of fitness-related behaviours with implications at the individual level (e.g., ability to feed properly and to avoid predators) but also in interactions with conspecifics (e.g., shoaling cohesion) and with other species. These PAR-induced swimming alterations may also hold ecological implications as behaviour is closely related to individual fitness and population persistence (Brodin et al., 2014).

These outcomes suggest PAR potential for neuroendocrine disruption (e.g., glucocorticoid signalling in the brain) leading to dysregulation of the stress axis. Considering the obtained results, it becomes highly relevant to perform additional studies to address the mechanisms involved in the observed responses and confirm the neuroendocrine disruption, particularly concerning glucocorticoid signalling pathways. This research could potentially help to identify and validate new biomarkers for SSRIs' neurobehavioural toxicity.

The protocol used in the present study constitutes a new methodological approach sensitive to screen for SSRIs' behavioural effects, allowing an integrative and robust study of zebrafish behaviour in different scenarios with significant ecological relevance.

CRediT authorship contribution statement

Carla S.S. Ferreira: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Cátia Venâncio:** Investigation, Writing – original draft, Writing – review & editing. **Mónica Almeida:** Investigation, Writing – review & editing. **Isabel Lopes:** Writing – review & editing. **Peter Kille:** Supervision, Writing – review & editing. **Miguel Oliveira:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Visualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgements

Thanks are due to the financial support to CESAM by FCT - Foundation for Science and Technology (UIDB/50017/2020 + UIDP/50017/2020+LA/P/0094/2020) and to FCT/MCTES for the financial support to the project NanoPlanet (DOI 10.54499/2022.02340.PTDC). Carla Melo was supported by FCT through a PhD Grant (2021.04580.BD) and Catia Venâncio is a contracted researcher under the scope of the NanoPlanet project (Ref.: CDL-CTTRI-141-SGRH/2023).

References

Adhish, M., Manjubala, I., 2023. Effectiveness of zebrafish models in understanding human diseases—a review of models. Heliyon 9 (3), e14557.

Almeida, A. R., Tacão, M., Machado, A. L., Golovko, O., Zlabek, V., Domingues, I., Henriques, I, 2019. Long-term effects of oxytetracycline exposure in zebrafish: A multi-level perspective. Chemosphere, 222, 333–344.

Ansai, S., Hosokawa, H., Maegawa, S., Kinoshita, M., 2019. Chronic fluoxetine treatment induces anxiolytic responses and altered social behaviors in medaka. *Oryzias latipes*. *Behavioural Brain Research*. 303, 126–136.

Arnnok, P., Singh, R.R., Burakham, R., Pérez-Fuentetaja, A., Aga, D.S., 2017. Selective ptake and bioaccumulation of antidepressants in fish from effluent-impacted Niagara River. Environ. Sci. Technol. 51 (18), 10652–10662.

Bertram, M.G., Martin, J.M., McCallum, E.S., Alton, L.A., Brand, J.A., Brooks, B.W., Cerveny, D., Fick, J., Ford, A.T., Hellström, G., Michelangeli, M., Nakagawa, S., Polverino, G., Saaristo, M., Sih, A., Tan, H., Tyler, C.R., Wong, B.B.M., Brodin, T., 2022. Frontiers in quantifying wildlife behavioural responses to chemical pollution. Biol. Rev. 97, 1346–1364.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Phil. Trans. R. Soc. B 369, 20130580.

de Farias, N.O., Oliveira, R., Moretti, P.N.S., e Pinto, J.M., Oliveira, A.C., Santos, V.L., Rocha, P.S., Andrade, T.S., Grisolia, C.K., 2020. Fluoxetine chronic exposure affects growth, behaviour and tissue structure of zebrafish. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. *237*, 108836.

Diaz-Camal, N., Cardoso-Vera, J.D., Islas-Flores, H., Gómez-Oliván, L.M., Mejía-García, A., 2022. Consumption and occurrence of antidepressants (SSRIs) in pre- and post-COVID-19 pandemic, their environmental impact and innovative removal methods: a review. Sci. Total Environ. 829, 154656.

Faimali, M., Gambardella, C., Costa, E., Piazza, V., Morgana, S., Estevez-Calvar, N., Garaventa, F., 2017. Old model organisms and new behavioural endpoints: swimming alteration as an ecotoxicological response. Mar. Environ. Res. 128, 36–45.

Ferreira, C.S.S., Soares, S.C., Kille, P., Oliveira, M., 2023a. Identifying knowledge gaps in understanding the effects of selective serotonin reuptake inhibitors (SSRIs) on fish behaviour. Chemosphere 335, 139124.

Ferreira, C.S.S., Venâncio, C., Kille, P., Oliveira, M., 2023b. Are early and young life stages of fish affected by paroxetine? A case study with *Danio rerio*. Sci. Total Environ. 900, 165706.

Ferreira, C.S.S., Venâncio, C., Oliveira, M., 2023c. Nanoplastics and biota behaviour: known effects, environmental relevance, and research needs. Trends Anal. Chem. 165, 117129.

Homberg, J.R., Olivier, J.D.A., Smits, B.M.G. Mul, J.D., Mudde, J., Verheul, M., Nieuwenhuizen, O.F.M., Cools, A.R., Ronken, E., Cremers, T., Schoffelmeer, A.N.M., Ellenbroek, B.A, Cuppen, E. Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience 2007, 146, 1662–1676.

Hossain, M.S., Kubec, J., Guo, W., Roje, S., Ložek, F., Grabicová, K., Randák, T., Kouba, A., Buřič, M., 2021. A combination of six psychoactive pharmaceuticals at environmental concentrations alter the locomotory behaviour of clonal marbled crayfish. Sci. Total Environ. 751, 141383.

Huang, I.J., Sirotkin, H.I., McElroy, A.E., 2019. Varying the exposure period and duration of neuroactive pharmaceuticals and their metabolites modulate effects on the visual motor response in zebrafish (*Danio rerio*) larvae. Neurotoxicol. Teratol. 72, 39–48.

Kleywegt, S., Payne, M., Ng, F., Fletcher, T., 2019. Environmental loadings of active pharmaceutical ingredients from manufacturing facilities in Canada. Sci. Total Environ. 646, 257–264.

Kreke, N., Dietrich, D.R., 2008. Physiological endpoints for potential SSRI interactions in fish. Crit. Rev. Toxicol. 37 (3), 215–247.

Martin, J.M., Saaristo, M., Bertram, M.G., Lewis, P.J., Coggan, T.L., Clarke, B.O., Wong, B.B., 2017. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. Environ. Pollut. 222, 592–599.

Martin, J.M., Nagarajan-Radha, V., Tan, H., Bertram, M.G., Brand, J.A., Saaristo, M., Dowling, D.K., Wong, B.B., 2020. Antidepressant exposure causes a nonmonotonic reduction in anxiety-related behaviour in female mosquitofish. J. Hazard. Mater. Lett. 1, 100004.

McDonald, M.D., 2017. An AOP analysis of selective serotonin reuptake inhibitors (SSRIs) for fish. Review Comparative Biochemistry and Physiology Part C 197, 19–31.

Muto, A., Taylor, M.R., Suzawa, M., Korenbrot, J.I., Baier, H., 2013. Glucocorticoid receptor activity regulates light adaptation in the zebrafish retina. Frontiers in Neural Circuits 7, 145.

Nowakowska, K., Giebułtowicz, J., Kamaszewski, M., Adamskic, A., Szudrowicz, H., Ostaszewska, T., Solarska-Dzięciołowskaa, U., Nałęcz-Jaweckib, G., Wroczyński, P., Drobniewska, A., 2020. Acute exposure of zebrafish (*Danio rerio*) larvae to environmental concentrations of selected antidepressants: bioaccumulation, physiological and histological changes. Comparative Biochemistry and Physiology, Part C 229, 108670.

Pelli, M., Connaughton, V.P., 2015. Chronic exposure to environmentally relevant concentrations of fluoxetine (Prozac) decreases survival, increases abnormal behaviours, and delays predator escape responses in guppies. Chemosphere 139, 202–209.

Saaristo, M., McLennan, A., Johnstone, C.P., Clarke, B.O., Wong, B.B.M., 2017. Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (*Poecilia reticulata*). Aq. Toxicol. 183, 38–45.

Sakamoto, T., Sakamoto, H., 2019. 'Central' actions of corticosteroid signalling suggested by constitutive knockout of corticosteroid receptors in small fish. Nutrients 11 (3), 611.

Salahinejad, A., Attaran, A., Meuthen, D., Chivers, D.P., Niyogi, S., 2022. Review - proximate causes and ultimate effects of common antidepressants, fluoxetine and venlafaxine, on fish behaviour. Sci. Total Environ. 807, 150846.

Salgado, R., Marques, R., Noronha, J.P., Mexia, J.T., Carvalho, G., Oehmen, A., Reis, M. A., 2011. Assessing the diurnal variability of pharmaceutical and personal care products in a full-scale activated sludge plant. Environ. Pollut. 159, 2359–2367.

Sumpter, J.P., Margiotta-Casaluci, L., 2022. Environmental occurrence and predicted pharmacological risk to freshwater fish of over 200 neuroactive Pharmaceuticals in Widespread use. Toxics 10, 233.

Venkatachalam, A.B., Levesque, B., Achenbach, J.C., Pappas, J.J., Ellis, L.D., 2023. Long and short duration exposures to the selective serotonin reuptake inhibitors (SSRIs) fluoxetine, paroxetine and sertraline at environmentally relevant concentrations Lead to adverse effects on zebrafish behaviour and reproduction. Toxics 11, 151.

Xie, H., Li, M., Kang, Y., et al., 2022. Zebrafish: an important model for understanding scoliosis. Cell. Mol. Life Sci. 79, 506.

Zhang, B., Chen, X., Pan, R., et al., 2017. Effects of three different embryonic exposure modes of 2, 2′, 4, 4′-tetrabromodiphenyl ether on the path angle and social activity of zebrafish larvae. Chemosphere 169, 542–549.