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Using a new high-throughput video-tracking platform to assess behavioural changes in *Daphnia magna* exposed to neuro-active drugs

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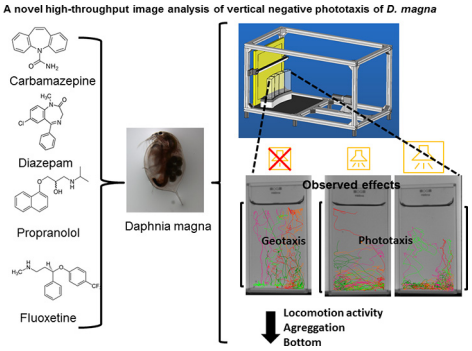
HIGHLIGHTS

- An image analysis system to study changes in the phototaxis of *D. magna* was developed.
- The neuro-active drugs diazepam, fluoxetine, propranolol and carbamazepine were tested.
- The drugs decreased the response of organisms to light and stimulated aggregation.
- Individuals presented reduced motility that tended to occur closer to the bottom.
- The tested drugs, except diazepam, induced the most severe behavioural effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Recent advances in imaging allow to monitor in real time the behaviour of individuals under a given stress. Light is a common stressor that alters the behaviour of fish larvae and many aquatic invertebrate species. The water flea *Daphnia magna* exhibits a vertical negative phototaxis, swimming against light trying to avoid fish predation. The aim of this study was to develop a high-throughput image analysis system to study changes in the vertical negative phototaxis of *D. magna* first reproductive adult females exposed to 0.1 and 1 μg/L of four neuro-active drugs: diazepam, fluoxetine, propranolol and carbamazepine. Experiments were conducted using a custom designed experimental chamber containing four independent arenas and infrared illumination. The apical-located visible light and the GigE camera located in front of the arenas were controlled by the Ethovision XT 11.5 sofware (Noldus Information Technology, Leesburg, VA). Total distance moved, time spent per zone (bottom vs upper zones) and distance among individuals were analyzed in dark and light conditions, and the effect of different intensities of the apical-located visible light was also investigated. Results indicated that light intensity increased the locomotor activity and low light intensities allowed to better discriminate individual responses to the studied drugs. The four tested drugs decreased the response of exposed organisms to light: individuals moved less, were closer to the bottom and at low light intensities were closer each other. At high light intensities, however, exposed individuals were less aggregated. Propranolol, carbamazepine and fluoxetine induced the most severe behavioural effects. The tested drugs at environmental relevant concentrations altered locomotor activity,

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geotaxis, phototaxis and aggregation in *D. magna* individuals in the lab. Therefore the new image analysis system presented here was proven to be sensitive and versatile enough to detect changes in diel vertical migration across light intensities and low concentration levels of neuro-active drugs.

1. Introduction

One of the major challenges that faces regulatory risk assessment today is to speed up the way of assessing threshold sublethal detrimental effects of existing and new chemical products. Recent advances in automated video/imaging allow to monitor in real time locomotor trajectories of individuals under a given stress and hence assessing multiple behavioural parameters in a relatively short time (Bownik, 2017). Behavioural responses are at the core of the adverse outcome pathway (AOP) concept that relates chemical exposure to subsequent molecular, cellular, physiological and behavioural changes that result in illness or injury to individuals (Ankley et al., 2010). The central nervous system (CNS) is the most complex organ that senses, processes and transmits information. Therefore, locomotor-based behavioural outputs of the CNS are highly sensitive measures of toxicant impact particularly for compounds with a neurodevelopmental or neurofunctional mode of action (Mora-Zamorano et al., 2018). Fong and Ford (2014) and Ford and Fong (2015) reported that antidepressant drugs induced phototaxis in amphipods, altered mobility of snails, memory, cognitive function and the ability to camouflage in cuttlefish at environmental relevant concentrations as low as pg-ng/L. More recently Rivetti et al. (2016) reported that psychiatric drugs such as the antidepressant fluoxetine, the anxiolytic diazepam and the neuropathic carbamazepine altered the negative phototaxis in the crustacean *Daphnia magna* at environmental relevant concentrations ranging from 1 to 1000 ng/L.

The ecotoxicological model crustacean species *D. magna* is a good candidate to study altered phototactic behaviour upon exposure to neuro-active drugs. *D. magna* share with vertebrates several of the neurotransmitters that are targeted by antidepressant and other neuroactive drugs. These include the presence of serotonin, dopamine, epinephrine and GABA receptor signaling pathways (Campbell et al., 2004; Campos et al., 2013; Ehrenström and Berglind, 1988; McCoole et al., 2012a; McCoole et al., 2012b; Weiss et al., 2012). *Daphnia* swimming behaviour is complex and hence precise of several measurement parameters. *Daphnia* moves with a characteristic hops generated by rhythmic beating of the second antennae pair (Dodson and Ramcharan, 1991). This means that cladoceran movement is not constant, it accelerates after the beat of the second antennae and subsequently the animal sinks when the second antennae return to the position to begin the next beating cycle. Therefore swimming speed depends on the movement characterized by accelerations followed by slowdowns. This parameter depends on *Daphnia* size (Hylander et al., 2014) and thus it is not always a reliable parameter to measure in ecotoxicological studies. Instead the distance moved by daphnids measured for a period of time may be a valuable swimming parameter indicating the locomotor activity. Some authors reported that this parameter may be altered by pesticides and neuroactive compounds (Bownik et al., 2018; Chevalier et al., 2014; Cooke, 1966; Hansen and Roslev, 2016; Zein et al., 2015). Additional parameters associated with the hop type movement that have been assessed in ecotoxicological studies are hopping frequency, swimming time or alternatively resting time between normal swimming (Bownik, 2017). Daphnia also have a collective behaviour termed warming, characterized by the aggregation of animals upon sensing light change, food presence or a predator pressure (Vollmer et al., 2006). Noss et al. (2013) reported increased warming behaviour upon exposure to titanium oxide nanoparticles.

One of the most ecological relevant swimming behavioural in *Daphnia,* however, is its phototaxis, which can be positive, negative or intermediate, depending of habitat characteristics, predation pressure and the clone (Cousyn et al., 2001; De Meester, 1993). Negative phototaxis is directly linked to diel vertical migration along the water column, which prevents *Daphnia* to be preyed upon fish during daylight (Cousyn et al., 2001; De Meester, 1993). Whereas negative phototaxis is a natural response of *Daphnia* to changing light conditions, it may be altered by toxicants. Most of the existing studies monitoring the negative phototactic behaviour in *Daphnia* across dark and light periods are based on manual monitoring of the relative position of animals (Cousyn et al., 2001; De Meester, 1993; Rivetti et al., 2016). The recent development of different video-tracking software in neuroscience research has enabled standardize and automate behavioural endpoints, promoting reproducibility and allowing for multiple endpoints to be recorded at once (Gómez-Canela et al., 2017). A complete system for high-throughput testing of the effect of pollutants on phototaxis in *Daphnia* requires (1) an observation chamber with special arenas for the video-tracking of the vertical position of daphnids, a background illumination based in infrared light, an apical-located source of visible light, and a GigE camera, and (2) an specific software for controlling the light stimuli (switch and intensity of the apical light and the camera) and for the video-tracking. Despite the increasing number of studies that have used automated video recording system to monitor *Daphnia* swimming behaviour (Bownik, 2017), few used infrared light for recording basal locomotion (Bahrndorff et al., 2016; Chevalier et al., 2014) and none combined both visible and infrared light to allow the simultaneous measurement of behavioural responses under dark and light conditions.

The aim of this study was to develop a complete high-throughput image analysis system specially designed to study changes in the vertical phototactic behaviour across varying light stimuli, including dark conditions of *D. magna* individuals exposed to neuroactive drugs. As a proof of concept, the effect of exposure of *Daphnia* to 0.1 and 1 μg/L of four neuroactive drugs (diazepam, fluoxetine, propranolol and carbamazepine) has been analyzed. Whereas these four drugs alter reproductive behaviour at low environmental relevant concentrations, only diazepam, fluoxetine and carbamazepine have been demonstrated to alter vertical phototaxic behaviour (Rivetti et al., 2016). Changes in locomotor activity, preferred area (bottom vs upper areas) and animal aggregation have been analyzed in groups of control and treated animals under consecutive periods of dark and apical light stimuli of different intensities.

2. Methods

2.1. Chemicals

Fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%), diazepam (CAS-No 439-14-5; analytical standard, purity 99%), carbamazepine (CAS-No 298-46-4; analytical standard, purity 99%) and propranolol hydrochloride (CAS-No 318-98-9; analytical standard, purity 99%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

2.2. Experimental animals

A single *D. magna* clone F, extensively characterized in previous studies (Barata and Baird, 2000) was used for all assays. Bulk cultures of 10 animals/L were maintained in ASTM hard reconstituted water (ASTM, 1994) as previously described (Barata and Baird, 2000). Bulk cultures were fed every other day with *Chorella vulgaris* Beijerinck 5 $\times 10^5$ cells/mL, corresponding to 1.8 μg C/mL (Barata and Baird, 2000).

The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14 h light: 10 h dark cycle and temperature at 20 ± 1 °C.

2.3. Chemical pre-exposure and behavioural assays

Changes in swimming behaviour were quantified by determining the response of groups of first-egg bearing adult females in the presence and absence of the tested chemicals. Prior to initiate behavioural assays organisms were exposed from birth (neonates <24 h old) until adulthood (when females carried the first clutch of eggs into their brood pouch, approx. 8 days at 20 °C) to 0.1 and 1 μg/L of fluoxetine (FX), diazepam (DZ), carbamazepine (CBZ) and propranolol (P). Previous studies indicated that the tested chemical concentrations altered reproductive parameters and/or phototaxis (Rivetti et al., 2016). Animals were exposed in groups of five individuals to the tested chemicals in 150 mL of ASTM hard water at the food ration of 5×10^5 cells/mL of *C. vulgaris*. The same concentration of ethanol 20 μL/L was used in all chemical-treatments as a carrier solvent and a solvent treatment was also included. Each treatment was replicated twice. The test medium was changed every other day until animals became adults and hence used for behavioural assays.

Two trials per treatment were performed in the same day. In each trail groups of five adult *Daphnia* from the experimental treatments were distributed among the four arenas (one arena per treatment) filled with 50 mL of ASTM. Treatments were randomized across chambers. Animals were then acclimated in dark conditions (only IR light on) for 5 min before video recording. For the behavioural analysis, the following experimental design was used: dark period/low intensity light period/dark period/high intensity light period/dark period. Total duration of each experiment was 25 min (5 min each period). Low and high intensity light were characterized by 84.5 and 48.7 lx and 2270 and 1330 lx on the top and the bottom of the water column, respectively. The selected light intensity ranges were similar to those measured from spring to summer in an oligotrophic lake inhabited by *Daphnia* species in Central Europe (Tilzer et al., 1995). After video-recording at 20 frames per second (fps), EthoVision XT 12 video tracking software was used for analysing the changes in the position of each animal. First of all, each arena was divided by half in two virtual zones, corresponding with the top and the bottom of the arena. Then, individual tracks of the five experimental animals in each arena were analyzed by using the social interaction module, determining the total distance moved (mm) and time spent in the bottom (s) for each animal. Moreover, the average distance (mm) among individuals in each arena was used as a measure of aggregation.

2.4. Complete high-throughput video tracking system

An experimental chamber for monitoring groups of *Daphnia* individuals simultaneously was designed (Fig. 1). Four experimental arenas were assembled in a horizontal rack. Each arena consisted in a large cell of 64 mL (8 × 4 × 2 cm, H × W × D) made in optical glass (7000-010-20-10; Hellma Analytics, Müllheim, German). An infrared backlight Elit 220 × 220 mm-IR 850-24 V-5 mm-emitting diode (LED) panel with a wavelength of 850 nm was placed behind the chambers to ensure homogeneous illumination of the arenas. A visible light LED strip (4000 K) of 25 cm mounted on the top of the arenas provided the light stimuli for the video-recording of the changes in behaviour during the light periods. Video-tracking was recorded by an uEye 5246-CP-GI-Mono-CMOS-GigE near infrared camera (IDS Imaging) with an optical 12 mm HR 2·2″ F1.45 lend and a resolution of 1280 × 1024 pixels positioned squarely 35 cm from the rack containing the experimental chambers. An IBP850 filter mounted to the camera only allowed to monitor infrared light. The visible LED strip and GigE camera were connected to a Mini USB-IO box and controlled by the Ethovision XT 11.5 software (Noldus Information Technology, Leesburg, VA). The exposure chamber was covered with an opaque polymer mask to avoid external light, and it was finally installed in a thermostated experimental room at 20 °C.

2.5. Chemical analyses

Stability of each compound during the tests was confirmed using solid-phase extraction and liquid chromatography-tandem mass spectrometry following Rivetti et al. (2016). Duplicated water samples of freshly made and old (48 h) test solutions were collected and pre-concentrated using Oasis HLB SPE cartridges (200 mg), conditioned with 10 mL of methanol followed by 10 mL of water. Five hundred mL of ASTM water were pre-concentrated at a flow rate of 10 mL/min and

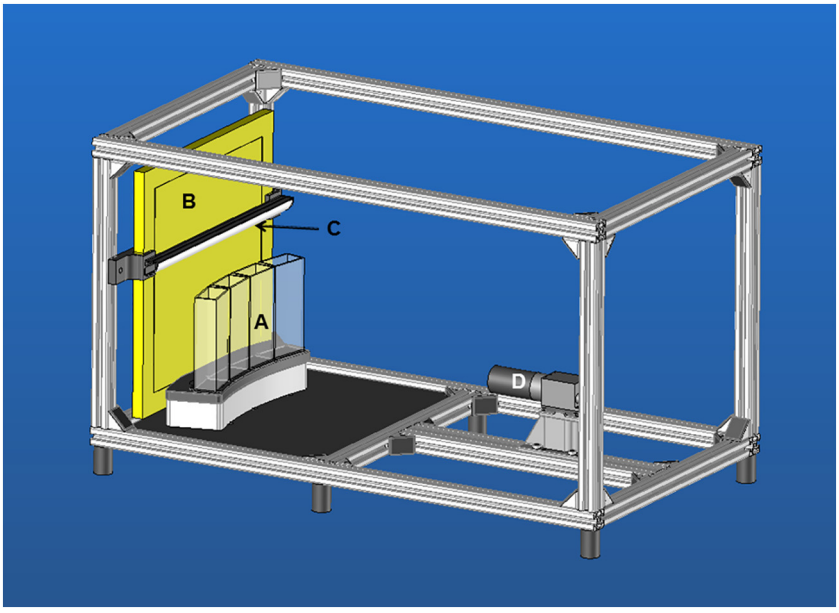

Fig. 1. Schematic representation of the vertical oriented four chamber behavioural device showing the four optical 64 mL glass cells (A), the infrared backlight diode infrared (LED) panel placed behind the cells (B), the visible light LED strip on the top of the cells (C) and the uEye 5246-CP-GI-Mono-CMOS-GigE near infrared camera positioned squarely 35 cm from the rack containing the experimental cells (D). Further details are described in the text.

eluted with 2 × 5 mL of methanol. The eluate was then reduced under nitrogen to almost dryness and reconstituted in 500 μL of methanol. All compounds were measured using LC-ESI-MS/MS (TqDetector, Acquity Waters, USA) following a previous study reporting an analytical method for simultaneous identification of a wide range of pharmaceuticals with minor changes (López-Serna et al., 2011). Separation was performed by using a Luna C18 column (150 mm × 2 mm ID, particle size 5 μm, Phenomenex, Torrance, USA) equipped with a SecurityGuard precolumn. The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A, then increased to 40% A in 5 min, 60% A in 10 min, reaching 100% A in 20 min and then return to initial conditions within 5 min. The system was operated at room temperature, the flow rate was set at 200 μL min^{-1} and 10 μL were injected. Fluoxetine, carbamazepine, diazepam and propranolol were analyzed under positive electrospray ionization mode (ESI+). Acquisition was performed in SRM mode using two transitions from [M + H] + precursor ion to daughter ions to identify each compound. The transitions used as well as the cone voltages and collision energies were in accordance with the above mentioned study (López-Serna et al., 2011). Quantification was based on external calibration standard 8 point curves (range between 0.5 and 1000 μg/L). Limits of detection and quantification (LD,LQ) defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were 1.35, 4.52 ng/L for fluoxetine; 0.15, 0.52 ng/L for diazepam; 0.07, 0.021 ng/L for carbamazepine and 0.02, 0.06 ng/L for propranolol. The data were acquired and processed using the MassLynx v 4.1 software package.

Measured residue levels of the tested concentrations in freshly prepared solutions (Table S1, 0 h) were pretty close to nominal values being in 6 out of 8 cases within 10% of nominal ones and having the max deviation of 29%. In all treatments measured concentrations of old test solutions were within 14% of freshly prepared ones (Table S1, 48 h). For the sake of clarity hereafter we will refer to nominal values.

2.6. Data analyses

Effects of the studied chemical treatments on measured behavioural parameters across and within experimental photoperiods (dark, low intensity light and high intensity light) were compared by two way ANOVA. Further treatment differences against control treatments were assessed by Dunnett's post hoc tests. Prior to analyses we ensured that the measured variables meet the ANOVA assumptions of normality and/or variance homoscedasticity (Zar, 1996).

3. Results

3.1. Behavioural responses

Results of the behavioural analyses are depicted in Figs. 2–4, which include temporal tracking responses of the studied individuals (graphs A) and overall ones across periods of dark and light (graphs B). The distance moved of experimental animals per min, which is a measure of locomotor activity, increased in the transition from dark to light conditions, and the magnitude of the increase in activity was dependent of the light intensity (Fig. 2A,B).

Exposure to propranolol resulted in a specific reduction of this photomotor response (Fig. 2B). Differences across photoperiods and of propranolol accounted for significant ($P < 0.05$) effects of illumination settings ($F_{2243} = 494.1$), treatment ($F_{8, 243} = 5.01$) and its interaction ($F_{16, 243} = 1.88$) in two way ANOVAs.

To analyse negative phototaxis in *D. magna* we determined the cumulative time that animals remained at the bottom of the arena relative to the total (%), which showed significant effects of illumination settings, treatment ($F_{8243} = 4.24$) and no interaction ($P > 0.05$; $F_{16,243} = 1.24$). Unexposed daphnids of the tested clone in darkness showed moderate levels of positive geotaxis, as 70% of time animals swam close to the bottom of the cells (Fig. 3A, B). Carbamazepine and the highest concentration of fluoxetine increased this positive geotaxis. Presentation of an apical light evoked a negative phototaxis, reflected by a significant increase ($P < 0.05$; $F_{2243} = 24.8$) in the time spent in the bottom of the arena during the light periods, this was especially evident at low light intensity. Interestingly, individuals exposed to low concentrations of carbamazepine, propranolol and high concentrations of fluoxetine exhibited an enhanced negative phototaxis within the low intensity light period (Fig. 3B).

The averaged distance among individuals was used as a measurement of aggregation. In unexposed daphnids, light induced aggregation in an intensity-dependent fashion. Effect of the tested chemical concentrations on aggregation varied across illumination settings. Under darkness the highest concentrations of carbamazepine, propranolol and fluoxetine increased aggregation; at low light intensity low levels of propranolol and both concentrations of fluoxetine increased

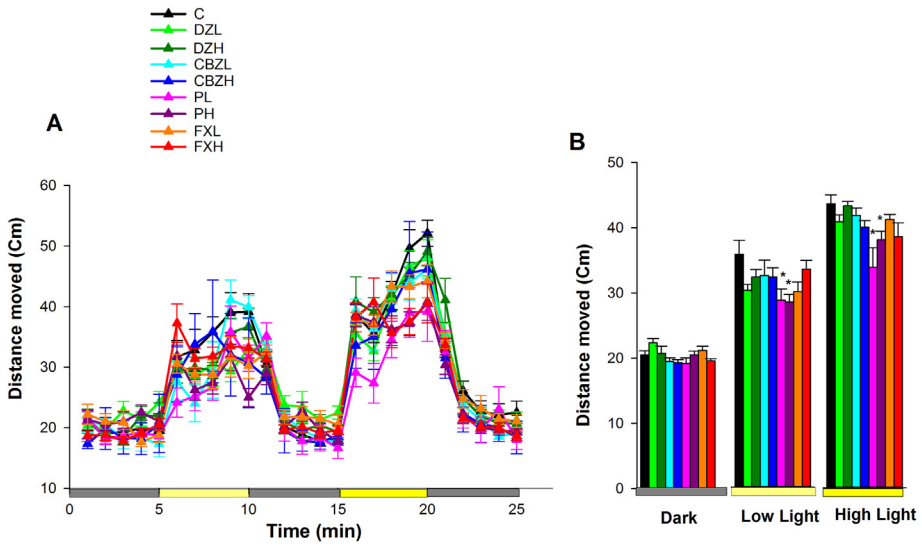

Fig. 2. Locomotor activity measured as the distance moved (Mean ± SE, $N = 10$, 5 animals per replicate) of exposed and unexposed *D. magna* individuals across consecutive 5 min periods of dark, low light intensity, dark, high light intensity and dark. Graphs A and B depict, respectively, the tracking responses for each min or across periods of dark and light.*indicates significant ($P < 0.05$) differences from control treatments following ANOVA and Dunnett's post hoc tests. C, DZP, CBZ, P, FX, L and H are respectively control, diazepam, carbamazepine, propranolol, fluoxetine, 0.1 and 1 μg/L treatments. Dark and light periods are depicted in the X axis.

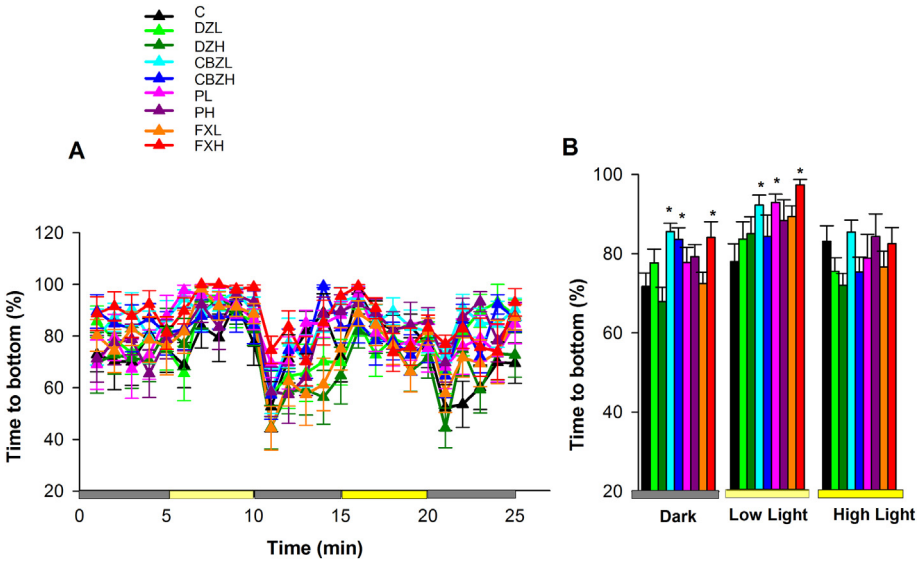

Fig. 3. Phototaxis measured as the cumulative time that animals remained at the bottom of the cells relative to the total (%) (Mean ± SE, $N = 10$, 5 animals per replicate) of exposed and unexposed *D. magna* individuals across consecutive 5 min periods of dark, low light intensity, dark, high light intensity and dark. Graphs A and B depict, respectively, the tracking responses for each min or across periods of dark and light.*indicates significant ($P < 0.05$) differences from control treatments following ANOVA and Dunnett's post hoc tests. Dark and light periods are depicted in the X axis. Abbreviations are described in Fig. 2.

aggregation; at high light intensities diazepam, high concentrations of carbamazepine and low concentrations of fluoxetine decreased aggregation. The previous results accounted for significant ($P < 0.05$) illumination settings ($F_{2243} = 34.29$), treatment ($F_{8243} = 7.17$) and interaction ($F_{16,243} = 4.43$) effects.

4. Discussion

We present in this manuscript a new complete high-throughput system for video-tracking groups of *Daphnia* in four arenas simultaneously. The platform allows to deliver light stimuli with different intensities from a position located above of the arenas. Moreover, by using the developed platform is possible to track the position of each animal in the water column at any time, allowing in this way to analyse basic behaviours like geotaxis, phototaxis and aggregation. As a proof of concept of the suitability of the developed platform in aquatic ecotoxicology, the effect of the exposure of *Daphnia* to environmentally relevant concentrations of four neuro-active drugs have been analyzed. Light intensity increased speed, negative phototaxis and aggregation of individuals from the tested *D. magna* clone, which is in line with previously reported negative phototaxic behaviour of this and other *D. magna* clones (Cousyn et al., 2001; De Meester, 1991; De Meester, 1993; Rivetti et al., 2016). Propranolol, carbamazepine and fluoxetine increased geotaxis under darkness and negative phototaxis at low light intensities. Propranolol and to a lesser extent the other tested drugs reduced locomotor activity of animals exposed to light. Increased geotaxis upon exposure to low concentrations of fluoxetine (i.e. 0.1 μg/L) agrees with previous results reported in amphipods but observed change in speed and phototaxis opposed (Bossus et al., 2014; Guler and Ford, 2010). In amphipods fluoxetine and other selective serotonin reuptake inhibitors (SSRI) increased the speed of animals under light and increased positive phototaxis (Bossus et al., 2014; Guler and Ford, 2010). Rivetti et al. (2016) also found that except propranolol, the tested drugs increased phototaxis in *D. magna*. Note, however, that in the previous study phototaxis was calculated using 10 discrete point measurements of the position of individuals relative to a higher intensity light source (500 Wm^{-2}, which was

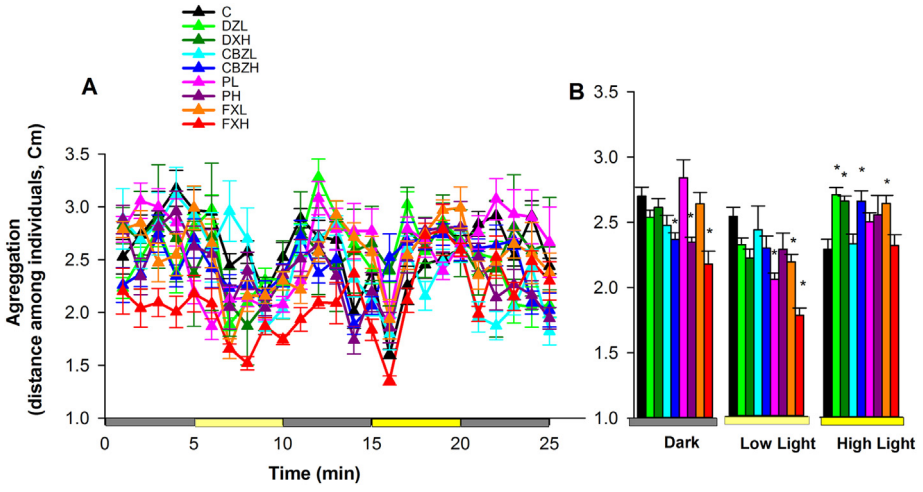

Fig. 4. Aggregation behaviour defined as averaged distance among individuals (Mean ± SE, $N = 10$, 5 animals per replicate) of exposed and unexposed *D. magna* individuals across consecutive 5 min periods of dark, low light intensity, dark, high light intensity and dark. Graphs A and B depict, respectively, the tracking responses for each min or across periods of dark and light.*indicates significant ($P < 0.05$) differences from control treatments following ANOVA and Dunnett's post hoc tests. Dark and light periods are depicted in the X axis. Abbreviations are described in Fig. 2.

equivalent to a 5350 lx at the surface and 3220 lx at the bottom), whereas our measurements were based on a continuous 5 min monitoring of the time spend in a relative position relative to a lower intensity light source (48.7–84.5 lx). Indeed in our study, at the highest light intensity (1330–2270 lx), exposed animals did not change their position to light relative to the control ones. Thus it is possible that at even higher light intensities than those used in the present study the studied drugs could act on phototaxis differently.

The studied light intensity ranges of the present work (40–2270 lx) were similar to those measured from spring to summer in an oligotrophic lake inhabited by *Daphnia* species in Central Europe (Tilzer et al., 1995). It is well known that negative phototaxis together with being smaller at first reproduction are fish anti-predator defence *Daphnia* mechanisms, and that these defences are more effective under low light intensities of 37–153 lx (Effertz and von Elert, 2014; Effertz and von Elert, 2017). At higher light intensities, fish predation efficiencies towards *Daphnia* preys are high and hence anti-predatory defences are less effective (Tałanda et al., 2018). This means that in the present study the higher reported effects at low than at high light intensity agree with reported cost-benefits of anti-predatory defences.

Aggregation behaviour has also been shown to reduce the vulnerability to predation. Predators dislike to attack aggregated prey (Allen, 1920; Neill and Cullen, 1974). Jensen et al. (1999) found that light, when it was heterogeneously distributed from the surface, enhanced aggregation. In our tested system light was attenuated by half from the top to the bottom of the experimental cells and aggregation in control treatments increased from darkness to low light intensity, thus our results agree with the previous study. On the contrary, in high intensity light conditions, the decrease in aggregation found in animals exposed to diazepam, high carbamazepine and low fluoxetine could increase the chances of *Daphnia* individuals to be predated by fish.

There is an increasing number of studies that have used video tracking devices to assess changes in *Daphnia* swimming behaviour upon exposure to chemicals (Artells et al., 2013; Bahrndorff et al., 2016; Barrozo et al., 2015; Bownik et al., 2018; Cano et al., 2017; Chevalier et al., 2015; Cruzeiro et al., 2017; Ferrario et al., 2018; Häder and Erzinger, 2017; Hansen and Roslev, 2016; Huang et al., 2017; Huang et al., 2015; Liu et al., 2018; Madeira et al., 2018; Nielsen and Roslev, 2018; Nikitin et al., 2018; Noss et al., 2013; Parolini et al., 2018; Ren et al., 2017; Ren et al., 2015; Stanley et al., 2016; Yang et al., 2018; Zein et al., 2014; Zhang et al., 2016). However, only few of them reported behavioural effects at environmental relevant concentrations far below those causing lethal or sublethal effects on stress markers or life-history traits (Nielsen and Roslev, 2018). Thus, many of the above mentioned studies may have falsely concluded that the tested chemicals have behavioural disrupting modes of action when in fact a much simpler explanation was not previously ruled out (e.g., caused systemic toxicity). This means that there is an urgent need for developing sensitive behavioural assays able to detect neurofunctional effects, which should occur at concentrations far below those causing any toxic response. Our results together with few other studies conducted in *Daphnia* (Nielsen and Roslev, 2018) provide an example that neuro-active drugs altered behavioural responses at environmental relevant concentrations. Concentrations of 12–540 ng/L of fluoxetine, the active ingredient of Prozac, in surface waters and effluents have been found in US (Kolpin et al., 2002). Concentrations of diazepam ranging from 4 to 40 ng/L have been found in Spanish urban rivers (Valcárcel et al., 2012). Carbamazepine is fairly persistent in water and hence can be found at concentrations ranging from 1 to up to 3000 ng/L in rivers receiving waste water treatment effluents (Muñoz et al., 2009; Tixier et al., 2003). Propranolol is also quite persistent in water and can be found at 10–60 ng/L in surface water (Bendz et al., 2005; Muñoz et al., 2009).

The observed behavioural effects of the studied drugs at ng/L are likely to be related to the disruption of neurofunctional processes of the central nervous system. The mechanisms of action of the SSRI fluoxetine on *D. magna* are better known than those of the remaining tested chemicals. Fluoxetine enhances brain serotonin activity in *Daphnia* (Campos et al., 2016), increases development and reproductive rates (Campos et al., 2012) and alters phototaxis. Recent studies using knockout *Daphnia* individuals lacking serotonin showed that these animals had the opposite phenotype as those exposed to fluoxetine: animals matured latter, reproduced less and were more mobile than wild type animals (Rivetti et al., 2018). There is thus a neurofunctional link between fluoxetine, its pharmacological target serotonin and effects (life-history and behavioural changes).

Whereas the pharmacological target of carbamazepine is to block voltage dependant sodium channels (Ambrósio et al., 2002), carbamazepine also increases extracellular serotonin levels (Lamichhane et al., 2014). Accordingly, carbamazepine may also act like fluoxetine, altering similarly behavioural responses to light. Diazepam decreases anxiolytic behaviour in fish and increases locomotion activity in decapod crustaceans, probably acting on GABA receptors (Ford and Fong, 2015; Whitman and Miller, 1982). Diazepam ameliorates also expressed anti-predatory life-history behaviour in *Daphnia* interacting with GABA (Weiss et al., 2012). Phototaxis is an adaptive anti-predatory behaviour (Cousyn et al., 2001) and hence could be also regulated by GABA and be affected by diazepam. Propranolol not only binds to β-adrenergic receptors but also to 5-HT1 receptors in humans acting as a serotonin receptor antagonist (Tierney, 2001). There is reported information that propranolol at low concentrations (0–1,1 μg/L) inhibits *Daphnia* swimming activity (Nielsen and Roslev, 2018), which is in line with our results.

In summary the four tested neuro-active drugs affected *D. magna* behaviour at environmental relevant concentrations and showed a response pattern that could be explained by reported neurofunctional mechanisms. Fluoxetine and carbamazepine acted on behaviour similarly probably through the activation of the serotonergic system. Propranolol was the only tested drug altering significantly ($P < 0.05$) locomotor activity, which was probably linked with reported antagonistic effects on serotonin receptors. Effects of diazepam were restricted to aggregation behaviour, which may be linked with its reported neurofunctional effects with GABA (Weiss et al., 2012).

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CRediT authorship contribution statement

Fátima C.P. Simão: Investigation, Writing - original draft. **Fernando Martínez-Jerónimo:** Investigation. **Victor Blasco:** Methodology. **Francesc Moreno:** Methodology. **Josep M. Porta:** Methodology. **João L.T. Pestana:** Conceptualization, Writing - review & editing. **Amadeu M.V.M. Soares:** Conceptualization, Writing - review & editing. **Demetrio Raldúa:** Software, Data curation. **Carlos Barata:** Conceptualization, Supervision, Writing - original draft.

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