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Phototactic behaviour and neurotransmitter profiles in two *Daphnia magna* clones: Vertical and horizontal responses to fish kairomones and psychotropic drugs

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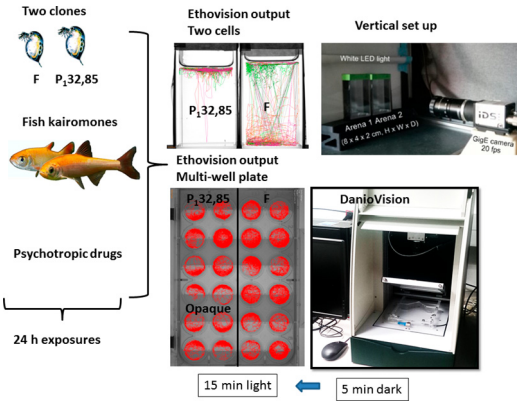
HIGHLIGHTS

- A multi-well plate set up was tested to monitor phototaxis in *Daphnia magna*
- Acrylic strips opaque to visible light covering half of wells were used.
- Two clones with opposed phototaxis and responses to fish were used.
- A multi-well plate set up was able monitor phototactic vertical and horizontal responses.
- Clonal phototactic differences were related to ddopamine and glycine levels.

GRAPHICAL ABSTRACT



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ABSTRACT

Animal behavioural responses are increasingly being used in environmental risk assessment. Nevertheless, behavioural responses are still hampered by a lack of standardisation. Phototactic behaviour in zooplankton and in particular in *Daphnia* has often been associated to vertical migration but there is also 'shore-avoidance' horizontal behaviour: *Daphnia* uses shades along the shore to swim either to or away from the shore and predators. Previously, we develop a vertical oriented behavioural hardware able to reproduce phototactic fish induced depth selection in *Daphnia magna*, its modulation by fish kairomones and psychotropic drugs and the neurotransmitter profiles associated to those responses. This study aims to test if it is possible to use an horizontal 24 multi-well plate maze set up to assess phototactic fish induced responses in *D. magna.* The study was conducted using two clones with opposed phototaxis upon exposure to fish kairomones and using psychotropic drugs known to modulate phototaxis. Acrylic strips opaque to visible light but not to the infrared one were used to cover half of the arena of each of the wells of the multi-well plate. Clone $P_1$32,85 showed positive phototaxis in either the vertical and horizontal set up and negative phototaxis when exposed to fish kairomones or to the muscarinic acetylcholine receptor antagonist's scopolamine and atropine. The opposite behaviour was observed for clone F. Diazepam and pilocarpine ameliorate fish kairomone induced negative phototaxis and picrotoxin increased it only in clone $P_1$32,85 in the vertical set up. The determination of neurotransmitters showed much greater concentrations of dopamine and of glycine in clone F, which may be relate to its negative phototaxis and its observed lower responsiveness to fish kairomones. The results from this study suggest a simple, fast, and high throughput phototactic behaviour assay for *D. magna* that can be easily adapted to other species.

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1. Introduction

Behavioural trait responses are increasingly being used in environmental risk assessment of chemicals (ERA) as they usually are more sensitive than lethality and faster to assess than growth, development and reproduction endpoints (Ford et al., 2021). Nevertheless, the use of behavioural responses in ERA is still hampered by a lack of standardisation (Ford et al., 2021). Recently the development of behavioural commercial equipment has minimized some of the problems of standardisation by providing a controlled environment within which to conduct behavioural assays (Pyle and Ford, 2017). A multi-well plate system is desirable as the plates have standardised dimensions, are readily available, and are compatible with commercial behavioural hardware (Bedrossiantz et al., 2020). In recent years, there have been some good examples of static, multi-well plate systems for a high-throughput measurement of swimming activity and locomotion mode changes across compounds that altered behaviour in crustaceans and fish (Bownik, 2017; Rennekamp and Peterson, 2015). Fish predators, pollutants and other environmental cues not only can alter the swimming speed and the mode of locomotion of crustaceans but also can modulate phototactic behaviour (Bedrossiantz et al., 2021; Pietrzak et al., 2017).

Changes in phototactic behaviour in response to chemical signals released from fish predators (i.e. fish kairomones) have been widely studied in the crustacean prey *Daphnia magna*, which is an established model organism in ecotoxicology and evolutionary ecology (Baird et al., 1998; Cousyn et al., 2001). *D. magna* populations adapted to high fish predation pressures show a marked negative phototactic behaviour when exposed to fish kairomones (De Meester, 1993) and this trait is highly heritable, which means that there is substacia genetic variation within and among populations (De Meester, 1996). Daphnia also shows 'shore-avoidance' behaviour: individuals use shades along the shore to "know" where the shore is (Gliwicz and Rykowska, 1992) or to avoid predators (Wicklum, 1999). *Daphnia* phototaxis is also sensitive to the direction and wavelength of light (De Meester, 1992). Thus, it is not surprising to find many different behavioural custom set ups and/or hardware devices that succeed in studying phototactic changes of *Daphnia* to olfactory cues from food, kairomones from competitors and predators, light wavelengths and temperature, among other factors (Dawidowicz and Loose, 1992; De Meester, 1991; Langer et al., 2019; Maszczyk, 2016; Pietrzak et al., 2017; Roozen and Lurling, 2001). Unfortunately, the above setup did not used commercially available behavioural equipment, thus limiting standardisation of phototactic behavioural assays and hence their use in ERA.

Other key premises that need to be addressed to allow the implementation of behavioural responses into ERA are the improvement of the reliability and reproducibility of behavioural end points and a better mechanistic understanding of contaminant- induced behavioural alterations (Ford et al., 2021).

In a previous work we developed a vertical oriented high-throughput image analysis behavioural hardware (VBH) able to measure both locomotor and vertical phototactic behaviour (Simão et al., 2019). This VBH was also able to monitor in a high-throughput and reproducible way *D. magna* phototactic changes to fish kairomones in a well-known responsive clone $P_1$32,85 (Bedrossiantz et al., 2021). The previous study also characterized the pharmacological modulation of phototactic behaviour to fish kairomones using cholinergic and GABAergic compounds (Bedrossiantz et al., 2021). Furthermore, the analysis of the profile of neurotransmitters and related metabolites showed that the *D. magna* behavioural responses induced by fish depend on changes in acetylcholine, dopamine and GABA (Bedrossiantz et al., 2021). Nevertheless, our VBH was only able to precisely monitor the trajectories of 10 to 12 adults of 15 days old from a single clone. It should be desirable to develop a phototactic horizontal multi-well plate system able to monitor younger organisms and a greater number of animals at the same time.

We previously developed a commercial 24 multi-well plate hardware to study short-term habituation of the escape response in 4 days old *D. magna* juveniles evoked by sudden changes in light intensity (Bedrossiantz et al., 2020). The aim of this study is to further develop the previously reported micro-well plate behavioural hardware (MWH) to monitor phototaxis using a simple maze design in which each well, hereafter named arena, was divided in two halves, one of them opaque to light. Furthermore, we also aimed to increase our current mechanistic understanding of phototactic responses considering both vertical and horizontal oriented responses in two clones of opposed behaviour, using selected psychotropic compounds known to affect phototaxis and by studying the neurotransmitters and associated metabolites in these two clones. Clones included a well-known one that strongly respond to fish kairomones changing its phototaxis from positive to negative (clone $P_1$32,85) (De Meester, 1993), and a lab clone F that shows negative phototaxis (Simão et al., 2019). The proposed MWH was tested using five compounds: scopolamine, which is known to change phototaxis mimicking fish kairomones (Bedrossiantz et al., 2021), diazepam, picrotoxin and pilocarpine, known to modify the effect of fish kairomones (Bedrossiantz et al., 2021), and atropine, a compound acting in the same way as scopolamine. The studied compounds are currently used as neuropathological treatments or/and are natural products that can be released to the environment, thus residue levels at μg/L and ng/L can be found in waste water and surface waters, respectively (de Almeida et al., 2015; Gomez-Canela et al., 2021; Jahn, 2001; Wang et al., 2017). For the purpose of this study we will use pharmacological doses of the above mentioned compounds.

2. Methods

2.1. Experimental animals

Two *D. magna* clones were used. On the one hand, a *D. magna* clone $P_1$32,85 obtained from two generations of intraclonal mixes within clone P_1, isolated from a small pond which contained fish (Driehoeksvijver, Heusden, isolated in August 1986). Clone $P_1$32,85 is known to become negatively phototactic in the presence of fish chemicals (De Meester, 1993). On the other hand, a laboratory clone F originated from England (Baird et al., 1991) that have been maintained in our lab for more than 30 years and that has negative phototaxis (Simão et al., 2019). Bulk cultures of 10 animals/300 mL or 50 individuals/1.5 L were maintained in ASTM hard water (ASTM, 1999) and were fed every day with 5×10^5 cells/mL of *Raphidocelis subcapitata*. Prior to experiments, cultures were maintained for three generations until adult females release their third brood and then were re-initiated with newborn individuals. To achieve the required number of experimental animals needed for behavioural tests, several larger cultures of 50 individuals/1.5 L were cultured and maintained for 4 and 15 days until used. These cultures were initiated from third brood neonates <24 h old. The culture medium was renewed three times a week and photoperiod was set to 16 h light:8 h dark cycle, and temperature at 20 ± 2 °C.

2.2. Chemical compounds

Five modulators of the cholinergic and GABAergic neurotransmitter systems were tested. These include the muscarinic agonist pilocarpine (PILO) and antagonists scopolamine (SCOP) and atropine (ATR); the GABA agonist diazepam (DZP) and antagonist picrotoxin (PCR). Purchased salts or pure compound suppliers are in Supplementary material.

2.3. Preparation of kairomone enriched media

To obtain fish kairomone water-borne compounds (FK), two 8 cm juvenile fish (*Leuciscus idus*) were allowed to swim in 15 L ASTM hard water for 24 h, after which was filtered using 0.45 μm glass fiber filters and several serial dilutions were tested to obtain a moderate exposure levels. A final dilution of 8 times was used for the study. This procedure was repeated for each experiment. This method was used to simulate fish predation risk (Bedrossiantz et al., 2021; De Coninck et al., 2013; De Meester and Cousyn, 1997). The kairomone concentration corresponded roughly to 1 fish in 60 L.

2.4. Experimental procedures

For each compound only a single concentrations was selected among those having the greatest modulatory effects on the phototactic behaviour obtained in a previous study (Bedrossiantz et al., 2021), with the exception of ATR, which was tested for the first time and its concentration was selected from preliminary assays. Tested concentrations were 1 μg/L for PCR, 100 μg/L for DZP, SCOP and 1000 μg/L for PILO and ATR.

Except DZP, whose stocks were prepared in ethanol, stocks for the rest of compounds were prepared in Milli-Q water on the day of the experiment. Final concentration of ethanol in DZP and control solvent solutions was 10 μL/L. Experimental treatments for each compound were defined as: control, FK (fish-kairomone conditioned water), the compounds alone or the mixture and the combination of both treatments. Chemical stability studies in ASTM water showed that all compounds were stable in water (Bellot et al., 2021).

D. magna females, either 4 days old juveniles or 15 days old adults, were pre-exposed to the selected treatments for 24 h, in groups of 12 juveniles or 5 adult individuals in 300 mL of test medium in 500 mL glass vessels prior to behavioural assays. We used 24 juveniles or 10–15 adults per treatment to get an acceptable level of replication. The pre-exposure period was chosen taking into account that after 3 h of FK exposure, it is possible to detect changes in daphnids behaviour (De Meester and Cousyn, 1997). Pre-exposures were conducted with food (5×10^5 cells/mL of *R. subcapitata*).

2.5. Behaviour swimming assay

Following exposures, the swimming tracks of individuals from clones $P_1$32,85 and F pre-exposed for 24 h to the studied treatments were assessed either using a vertical experimental chamber hardware (VBH) (Bedrossiantz et al., 2021) or an horizontal 24 multi-well plate maize one (MWH). The VBH has been described elsewhere (Bedrossiantz et al., 2021; Simão et al., 2019). Briefly, it includes two independent arenas of 64 mL (8 × 4 × 2 cm, H × W × D), a backlight infrared illumination and an apical-located LED stripe producing visible white light controlled by EthoVision XT software (RRID: SCR_000441). As described elsewhere (Bedrossiantz et al., 2021; Simão et al., 2019) the apical visible light creates a light gradient in the water column of the arena thus positive and negative phototactic individuals are positioned close to the top and bottom of the arena. For each compound and FK combination, three behavioural trials were performed. In each trial, groups of 5 *Daphnia* from two different treatments were distributed among the two arenas filled with 50 mL of test solution without food. This means that a total number of 15 individual/replicates were monitored per treatment. Treatments were randomized across chambers. Animals were then acclimated in the dark for 10 min before video recording. For the behavioural analysis, animals were recorded in the dark (5 min), and under a moderate intensity apical white light (375 lx, 15 min). After video-recording at 20 frames per second (fps), EthoVision XT 14 video tracking software was used to analyze the changes in the position of each animal. First of all, each arena was divided in three identical zones, corresponding with the top, middle 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 of the software, determining the time spent in the top virtual zone (%). For statistical analyses for each individual the mean value of the last ten minutes of light period was considered.

For the MWH, animals were tracked in a DanioVision Observation Chamber™ for 20 min, with a 5 min dark phase followed by a 15 min light phase (50% intensity, 290 lx). In this video-tracking system, a GigE camera is located on the top of the chamber, allowing the use of different commercial multi-well plates in a holder mounted above the illumination system, a visible and infrared light source panels that irradiate the plate from below. A series of custom acrylic strips (DanioVision CorningCostar 3524 dark/light grid) situated below the plates between them and the light sources were used to provide a light and dark halves of the arena (Fig. S1, Supplementary material). The strips consisted of a black acrylic which was only transparent to infra-red light. During the dark phase, the entire arena was dark and, during the light phase one half of the arena was illuminated whilst the other half remained dark and *D. magna* could choose to be in either the light or dark side of the arena. It was expected that during dark phases animals would use all of the arena equally. During light phase, animals would exhibit phototaxis, showing a preference for either the light or dark side of the arena. Groups of 12 *D. magna* 4-day old juveniles pre-exposed to the each individual compound were distributed randomly to 24-well plates (one animal per well, two treatments per plate, two trials per compound). Animals did not feed throughout the behavioural assays. Videos were recorded at 30 frames per second and phototaxis was analyzed as the % of time that each individual was in the light zone. For statistical analyses for each individual we computed the mean value of over the last ten minutes of light period. Values were determined per min.

2.6. Analysis of neurotransmitters and related metabolites

We studied monoaminergic neurotransmitters and related metabolites in pools of five 4 days old juveniles of the two studied clones (5 replicates) cultured as those for behavioural tests following previous procedures (Fuertes and Barata, 2021). Individuals were sampled and frozen in liquid N_2 and maintained at −80 °C until analysis by ultra-high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer equipped with an electrospray (ESI) source (Xevo, TQS micro, Waters, Milford, MA, USA). Chromatographic conditions were based in a previous published paper based on the analysis of neurotransmitters and related metabolites in different brain areas of zebrafish (Mayol-Cabré et al., 2020). More details about the chromatographic conditions are reported in "*1.2.3. LC-MS/MS* analysis" of the Supplementary material (SM). In the present study, we determined up to 17 metabolites belonging to five different neurological signalling pathways as shown in Table S1, SM.

Protein levels were also measured using the Bradford method. Final results were reported relative to mg protein. Methods are provided in SM.

2.7. Data analyses

The behavioural experimental design followed a three-way ANOVA design with FK, compound treatments and clone as fixed factors. For the VBM we could have consider also the arena holding the animals as a nested factor but for the sake of clarity we keep the analysis simpler (Bedrossiantz et al., 2021). Prior to analyses the behevioral responses, percentadge of individual on the top virtual zone for the vertical device and on the light zone for the horizontal device, and metabolite concentration data and residuals were tested to meet ANOVA assumptions of normality and variance homoscedasticity and if need it was arccosine (for %) or log transformed. Following ANOVAs, differences among treatments were further compared using post-hoc Tukey's multiple comparison tests. Analyses were performed with the IBM SPSS Statistics software v27.

3. Results

3.1. Phototactic behaviour

The already validated VBH was able to monitor the contrasting phototactic trajectories of the adult females of the two studied clones. Full trajectories of the phototactic time responses are in Figs. S2, S3 of Supplementary material. Adults of clone $P_1$32,85 were located predominantly at the top of the arena increasing their percentage of occurrence from 60% during the dark period until 100% at the end of the light one (Fig. S2 top graph panel). Alternatively, individuals from clone F tended to remain closer to the bottom under dark and light conditions having a percentage of occurrence in the top virtual zone <50% (Fig. S2 bottom graphs panel). This is clearly observed in Fig. 1 that depicts the % of time in the upper zone for the last 5 min of light (Graphs A–E, white and white/strike bars for clones $P_1$32,85 and F, respectively). An equivalent behaviour was observed in the MWH in which the proportion of individuals from clone $P_1$32,85

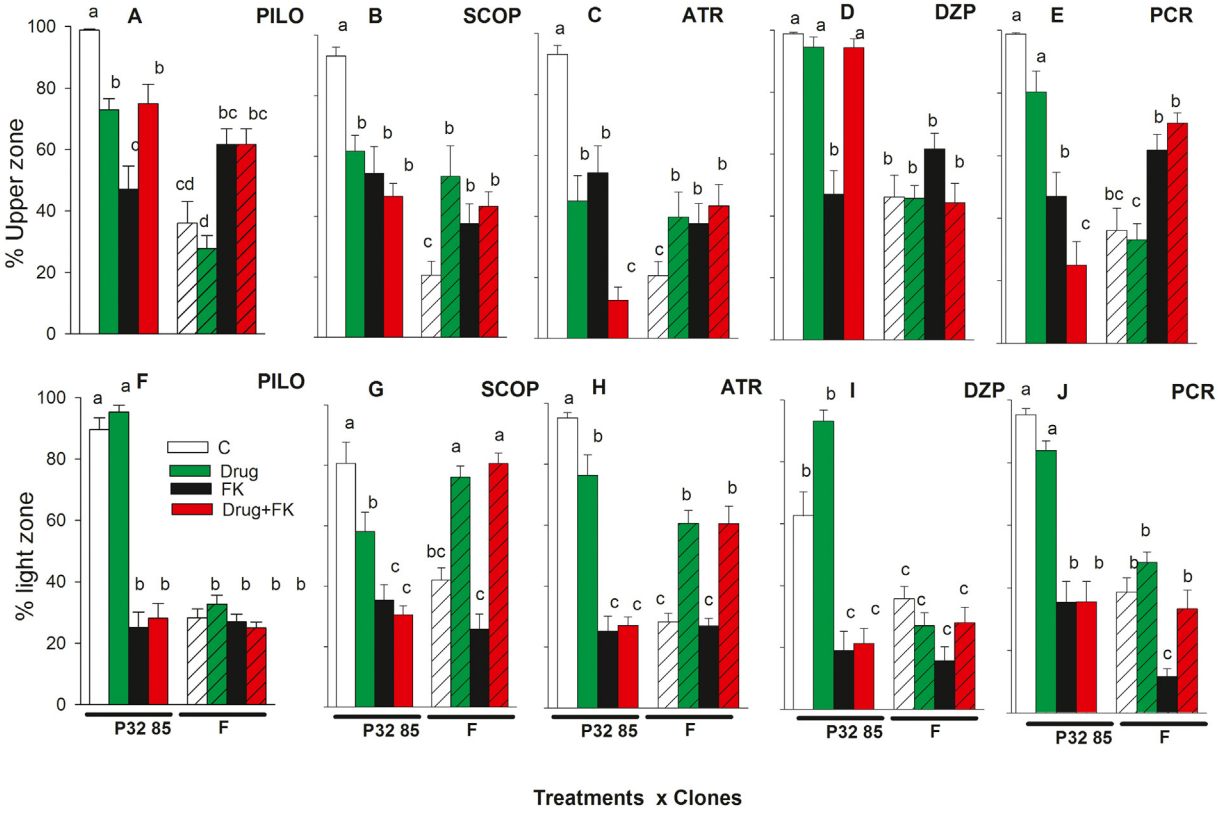

Fig. 1. Percentage of individuals located in the upper zone in the vertical hardware and in the light zone in the 24 multi-well plate set up during the last 5 min of light. Upper graphs correspond to the vertical oriented set up and lower ones to the horizontal 24 multi-well one. Results are the means and SE of 15–24 individuals. Different letters above bars indicate significant differences across treatments and clones after ANOVA and Tukey HSD post-hoc test. Treatments were FK, fish kairomones; SCOP, scopolamine 100 μg/L; ATR, atropine 1 mg/L, DZP, diazepam 100 μg/L; PCR, picrotoxin 1 μg/L; PILO, pilocarpine 1 mg/L. Open and crosshatching bars represent the different clones.

located in the light zone increased from 50% during dark to 100% during light (Fig. S3, top graph panel), whereas in clone F this proportion tended to decrease (Fig. S3, bottom graph panel). This resulted in a high % of time remaining in the light zone for the last 5 min of light in clone $P_1$32,85 relative to clone F (Fig. 1, Graphs F to J, white and white/strike bars for clones $P_1$32,85 and F, respectively).

Results for the three-way ANOVA testing the effects of FK, drug treatments and clone in the observed phototactic trajectories in both the vertical and horizontal devices denoted also contrasting effects of clones in all comparisons (significant, $P < 0.05$, effects of clone alone or its interaction with the other factors, Table 1). Fish kairomones (FK) decreased significantly the % of individuals remaining in the upper or light zone in clone $P_1$32,85 but only did so in 1 out of 12 comparisons in clone F. More specifically in clone F, FK either increase the % of individuals remaining in the upper zone (Fig. 1B, C) or it has hardly any effect (Fig. 1, rest of graphs). These contrasting effects of FK across clones explained the significant FK x Clone interaction (Table 1).

In the VBH, the tested drugs behave in a similar way as in a previous study in clone $P_1$32,85 (Bedrossiantz et al., 2021): SCOP decreased positive phototactic behaviour, PCR did so only when co-exposed with FK, whereas PILO and DZP diminished the effect of FK. Interestingly ATR, which has the same mode of action as SCOP, produced similar effects decreasing positive phototaxis (Fig. 1A–E). Effects of SCOP and ATR in clone F were opposed to those of clone $P_1$32,85, since increased positive phototactism irrespectively of FK. The remaining drugs (PILO, DZP, PCR) did not affect the phototactism of clone F in control or FK treatments. In the horizontal maze hardware only SCOP and ATR behave similarly in both clones as in

Table 1
Three-way ANOVA testing for the effects of clone, fish kairomones (FK), the tested drugs and their interaction on phototactic behaviour (% of individual on the top virtual zone for the vertical device and on the light zone for the horizontal device). Only degress of freedom (df), Fisher's coefficient (F) and *P* values are reported.

	Clone			FK			T			Clone × FK			Clone × T			FK × T			Clone × FK × T		
	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P
Vertical																					
PILO	1,96	40.6	<0.001	1,96	0.0	0.989	1,96	1.1	0.287	1,96	42.9	<0.001	1,96	1.8	0.18	1,96	22.7	<0.001	1,96	5.5	0.02
SCOP	1112	31.6	<0.001	1112	6.8	0.011	1112	0.0	0.966	1112	11.5	<0.001	1112	18.6	<0.001	1112	0.0	0.86	1112	8.1	0.01
ATR	1112	11.3	<0.001	1112	7.2	0.009	1112	11.8	0.001	1112	23.8	<0.001	1112	37	<0.001	1112	0.1	0.71	1112	1.1	0.3
DZP	1,90	80.5	<0.001	1,90	6.1	0.015	1,90	2.7	0.101	1,90	18.8	<0.001	1,90	15.9	<0.001	1,90	5.1	0.03	1,90	20.2	<0.001
PCR	1,90	3.3	0.07	1,90	7.3	0.008	1,90	4.5	0.037	1,90	107.1	<0.001	1,90	7.9	0.01	1,90	2.9	0.09	1,90	4.6	0.04
Horizontal																					
PILO	1184	161.6	<0.001	1184	204.2	<0.001	1184	1.3	0.256	1184	155.0	<0.001	1184	0.4	0.53	1184	0.9	0.35	1184	0.1	0.72
SCOP	1172	2.2	0.14	1172	40.2	<0.001	1172	21.3	<0.001	1172	21	<0.001	1172	76.3	<0.001	1172	8.4	<0.001	1172	0.1	0.82
ATR	1181	15.5	<0.001	1181	99.7	<0.001	1181	16.3	<0.001	1181	95.2	<0.001	1181	46.9	<0.001	1181	3.3	0.07	1181	2.6	0.11
DZP	1123	36.6	<0.001	1123	82.7	<0.001	1123	6.1	0.015	1123	42.5	<0.001	1123	4.0	0.05	1123	0.2	0.63	1123	11	<0.001
PCR	1146	73.9	<0.001	1146	119.1	<0.001	1146	2.1	0.153	1146	23.4	<0.001	1146	9.5	<0.001	1146	3.0	0.09	1146	0	0.96

the vertical one: diminishing positive phototactism in clone $P_1$32,85 and increasing it in clone F. The remaining three drugs PILO, DZP and PCR hardly have any phototactic effect when co-administered with FK. The above-mentioned drug mediated effects accounted for the significant clone x T or/and Clone x T x FK interactions in Table 1.

3.2. Protein and metabolites

Individuals of clone F were bigger than those of clone $P_1$32,85 and had higher protein levels (Fig. 2A). Four of 17 metabolites varied significantly between clones. Clone F has greater levels of dopamine (DA) and glycine (Gly) (Fig. 2B, E) and clone $P_1$32,85 higher levels of 3-Methoxytyramine (3MT) and GABA (Fig. 2C, D).

4. Discussion

Phototactic behaviour in zooplankton and in particular in *Daphnia* has often been associated to vertical migration but there is also 'shore-avoidance' behaviour: *Daphnia* uses shades along the shore to swim either to or away from the shore - this is how they "know" where the shore is (Gliwicz and Rykowska, 1992). Furthermore, high predation risk in the littoral neighbourhood may be considered an ultimate factor behind the evolution of shore avoidance behaviour in zooplankton (Wicklum, 1999). In this study, we tested for both types of behaviour using two clones having contrasting phototaxis. The results obtained in our study denoted similarities between both behaviour responses and quite contrasting responses of both clones. Fish kairomones and the muscarinic antagonistic drugs SCOP and ATR behave similarly decreasing positive phototactism in clone $P_1$32,85 in the vertical and horizontal oriented behavioural hardware (VBH and MWH, respectively). The muscarinic agonists PILO and the GABAergic compounds, DZP and PCR, only modulate FK effects on the phototactism in the VBH. The results obtained for the VBH for clone $P_1$32,85 are similar to a previous study (Bedrossiantz et al., 2021), thus confirming the robustness of FK responses of clone $P_1$32,85. Moreover, for clone $P_1$32,85 the MWH was also able to equally assess changes in phototactism induced by FK or by drugs such as SCOP and ATR. These are interesting results since apparently contradict previous studies that found that *D. magna* juveniles had a less marked phototactism to fish kairomones and psychotropic drugs than adult stages (De Meester and Dumont, 1988; Rivetti et al., 2015). Nevertheless, the previous studies were performed using different set ups, which may account for such differences. Furthermore, our 4 day old juveniles were at the pre-adolescent instar with well-developed ovaries that were nourishing the first clutch of eggs (Barata and Baird, 1998; Campos et al., 2018; Ebert, 1994). This means that our "juvenile" stage could behave as adults. The MWH has a greater high throughput capacity than the vertical one since it allows to simultaneously monitor phototactism up to 24 organisms of 4 days old whereas the latter device is only able to do so for 10–12 adults (Bedrossiantz et al., 2021). Furthermore, in the MWH each well contains a single individual, which makes statistical analyses simpler. It is difficult, however, to explain the failure of PILO and GABAergic drugs modulating phototaxis in the MWH. This is unlikely to be related with geotaxis since PILO and GABAergic drugs alone did not change phototaxis neither geotaxis in the vertical VBH. Previous studies found that *D. magna* has a distinct sensitivity for lateral and stray light colours, which is likely to be used to search for food and avoid fish predation, respectively (De Meester, 1992). Despite of using white illumination, light trajectories differed in both hardware: in the horizontal hardware (MWH) light come from below illuminating the whole arena except the opaque maze covered part, whereas the vertical hardware (VBH) has an apical light whose intensity decrease from the top to the bottom of the cuvette (Bedrossiantz et al., 2021). The direction of the light stimuli did not change the response to FK, SCOP or ATR in both hardware set ups, thus *Daphnia* equally perceive the risk of fish predation whatever the location of the light stimuli. Nevertheless, the modulation of the perception of FK by PILO and GABAergic drugs could be affected by the direction of light. In summary our results indicate that irrespectively of the direction of light FK or muscarinic antagonist affected phototaxis. On

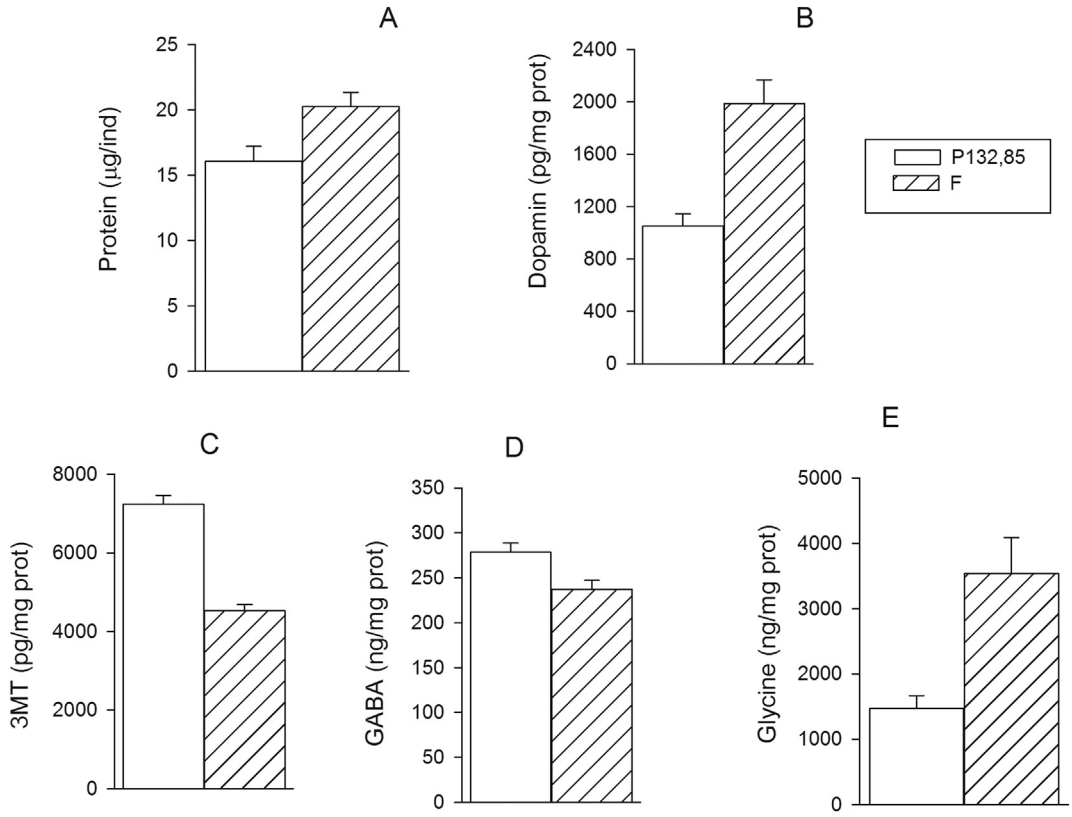

Fig. 2. Protein levels and selected neurotransmitters and related metabolites whose concentrations varied significantly ($P < 0.05$) between clones $P_1$32,85 and F. The remaining metabolites are in Table S4. Metabolite abbreviations are in Table S1 (Supplementary material).

the other hand, the modulation of FK effects could be related to light coming from above as it naturally occurs during daylight in a pond or a lake. Further research is needed to be conducted to test the last argument.

Results of SCOP and ATR in both hardware set ups and of FK in the vertical one in clone F were opposed to those of clone $P_1$32,85. SCOP and ATR increased positive phototaxis and FK in the vertical hardware also tended to do so in clone F. Clone F had a maladaptive phototactic responses to FK but such contrasting effects have already been reported in other clones. Boersma et al. (1998) studied the way different traits responded to fish kairomones across *D. magna* clones derived from different habitats with and without co-occurring fish and found that clones differed widely in their response to predator kairomones. None of the clones showed a response in all traits, clones from fish habitats were slightly more responsive to the presence of fish kairomones with some clones showing the opposite behaviour. Thus, our results agree with the previous study. The more responsive clone $P_1$32,85 come from an habitat with fish (De Meester, 1993), whereas we do not know the origin of clone F (Baird et al., 1991), which is likely to come from a fishless pond (Donald Baird personal communication).

In an attempt to study the apparent contrasting clonal effects of FK and of muscarinic agonists that can mimic it, we study how muscarinic related metabolites (i.e. cholinergic ones) varied across clones. Previous work showed that FK and SCOP decreased acetylcholine (Bedrossiantz et al., 2021). We also included other metabolic pathways that were de-regulated upon exposure to FK such as the dopaminergic, adrenergic, GABAergic and serotonergic ones.

Individuals of both clones have similar concentrations of acetylcholine. Interestingly clone $P_1$32,85 has much lower concentration of dopamine and greater levels of its metabolite 3-MT than clone F, which means that the latter clone has greater turnover rates of dopamine. Previous work showed that FK and SCOP increased the turnover rates of dopamine, thus having constitutively high levels of dopamine may hamper clone F to respond properly to FK and muscarinic antagonists (Bedrossiantz et al., 2021). GABAergic activation or inhibition by drugs or exogenous GABA have been related to prevention or shifts in phototactic and other life-history responses to FK in *Daphnia*, respectively (Bedrossiantz et al., 2021; Weiss et al., 2012). The previous argument apparently contradicts the greater levels of endogenous GABA found for the more responsive clone $P_1$32,85. Nevertheless, differences in constitutive GABA between the two studied clones, although significant different, were less than 15%, whereas that found between drug treatments that prevented phototaxic shifts to FK were close to 50% (Bedrossiantz et al., 2021). One of the most outstanding results was the almost three fold greater levels of constitutive glycine levels found in individuals of clone F. In *Drosophila* glycine depletion renders the circadian network susceptible to environmental changes (Frenkel et al., 2017) and GABA/Glycine receptors are involved in the *Drosophila* innate light preference (Zhao et al., 2019). This means that the much greater levels of Gly of clone F could be behind its opposed "innate" phototaxis.

5. Conclusion

In summary, our results showed that the high-throughput horizontal 24 multi-well plate maze set up (MWH) was able to measure in a similar way as the more ecological relevant vertical oriented hardware (VBH), the contrasting phototactic patterns of the two studied clones. Both set ups equally measured the opposed phototaxis of clones $P_1$32,85 and F, their response to the muscarinic agonists (atropine and scopolamine) and to fish kairomones. On the contrary, the horizontal set up failed to reproduce the phototactic responses obtained by VBH for the GABAergic compounds co-administered with fish kairomones in clone $P_1$32,85. It is unlikely that this failure was related to gravity since alone GABAergic compounds did not affect geotaxis in the VBH. Phototaxis in *Daphnia* can be vertical but also horizontal when is related with 'shore-avoidance' behaviour. Therefore, regulation of both phototactic responses is likely to involve similar neurological pathways. Indeed, in the copepod *Calanus finmarchicus* an horizontal set up was successfully used to investigate how the intensity and wavelength of light trigger vertical migration during the boreal night (Miljeteig et al., 2014). This means that the use of horizontal migration phototaxis set ups and small water volumes like our MWH can be used to study ecologically relevant phototactic behaviors related to either vertical or horizontal migration in zooplanktonic species.

CRediT authorship contribution statement

M.B; Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing; **C.G.-C.**; Supervision, review & editing; **C.B**; Supervision, Conceptualization, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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.scitotenv.2022.154684.

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