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Neurochemical exposure disrupts sex-specific trade-offs between body length and behaviour in a freshwater crustacean

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ABSTRACT

Ongoing pollution of aquatic ecosystems with neurochemical compounds warrants an improved understanding of how this affects key organisms. Neurochemicals are shown to alter the behaviour of common study species but it remains difficult to translate these results to biologically meaningful predictions across taxa. This is partly because studies on species with non-generic life-history strategies such as many freshwater crustaceans are currently underrepresented. Here, we use a laboratory experiment to assess baseline behavioural variation (spontaneous activity level and geotaxic behaviour) in the freshwater fairy shrimp *Branchipodopsis wolfi* and how this is affected by chronic exposure to an environmentally-relevant concentration of the anxiolytic pharmaceutical fluoxetine. The more conspicuously coloured and larger females of the species were overall less active and more benthic than males. Moreover, amongst females, vertical activity was negatively associated with size, while an opposite relationship was found for males. These trade-offs are likely part of an antipredator strategy to reduce the probability of being detected by visual hunters, but disappeared after exposure to fluoxetine. This is of particular interest since it is an effective proof of principle that neurochemicals may impact ecologically-relevant trade-offs between conspicuous morphology and antipredator behaviour. In natural ecosystems, such disturbed antipredator behavioural responses could have far-reaching fitness consequences.

1. Introduction

Animal behaviour is an organism-level integrative response to a range of internal and external stimuli (Levitis et al., 2009) and may contribute directly or indirectly to an individual's reproductive success and survival (Saaristo et al., 2018; Wong and Candolin, 2015). In recent years, neuroactive compounds increasingly contaminate natural ecosystems and profoundly affect wildlife behaviour (Brodin et al., 2014; Thoré et al., 2020b). They typically occur in the environment in the mid to low ng/L range (Puckowski et al., 2016), and act already at low doses on evolutionary conserved target molecules that control various physiological and behavioural systems (Gunnarsson et al., 2008; Mcdonald, 2017). Although there are ample studies on how neurochemicals affect wildlife, the exact ecological risks of neurochemical pollution still require further investigation. This is mainly because our capacity to extrapolate results across studies and taxa is still limited (Sumpter et al., 2014), and because we lack a comprehensive behavioural baseline information for many organisms (Tanoue et al., 2019; Thoré et al., 2021b).

Risk-assessment of pollutants typically starts with standardised laboratory trials and subsequent extrapolation (Brady et al., 2017; Segner and Baumann, 2016). This approach is strongly dependant on suitable study organisms, which ideally are easy to breed under laboratory conditions and are amenable to experimental manipulation (Fields and Johnston, 2005; Thoré et al., 2020a, 2021b). In this regard, fish such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) are popular study organisms. Because many toxicity mechanisms are evolutionary conserved, responses to chemical compounds are often similar across fish species (Gunnarsson et al., 2008; Villeneuve et al., 2014). Moreover, their physiological attributes are usually well-characterised and facilitate extrapolation across vertebrate taxa (Margiotta-Casaluci et al., 2014; Thoré et al., 2020a). Still, focussing on a limited number of model species with generic life-histories may result in a poor understanding of how chemical exposure affects the behaviour of a variety of other organism groups, including invertebrate taxa (Eagles et al., 2021).

Roughly 98% of all animal species are invertebrates, which often

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have physiological attributes and life-history strategies that are rare or even entirely absent in vertebrates (Kralj-fišer and Schuett, 2014). For instance, across the globe, freshwater fairy shrimps (Crustacea, Branchiopoda, Anostraca) are dominant competitors in temporary waters and are crucial for the ecological functioning of these habitats (Brendonck et al., 2017; Yang and Park, 2017). Specifically, they play a key role in food webs as primary consumers that graze on phytoplankton and help suppress harmful algal blooms (Yang and Park, 2017). In turn, fairy shrimps are regularly preyed upon by birds and invertebrate predators (Brendonck et al., 2017, 1995; Horváth et al., 2013). Like many other aquatic species from temporary waters, fairy shrimps are adapted to deal with the periodic drying of their habitats by producing drought-resistant eggs that lay dormant in the sediment until the next inundation (Brendonck et al., 2017; Brendonck and De Meester, 2003). Moreover, they typically grow and mature rapidly in order to complete their life-cycle before drying, and are adapted to strong daily fluctuations in environmental conditions such as pH (Brendonck et al., 2017; Pinceel et al., 2017a). While their resilience to natural stressors may well trade-off with their sensitivity to anthropogenic pollutants (Forbes and Calow, 2002; Lahr, 1997), this remains largely unstudied.

Fairy shrimps are interesting as behavioural models for aquatic invertebrates with similar life-histories and to increase the resolution of ecological risk assessment. Also, because of their fast life-cycle and high reproductive output, they are ideally suited to study the impact of whole-life and multigenerational exposure to (neuro)chemicals within a relatively short timeframe. So far, however, fairy shrimp behaviour has hardly been studied and information is mostly confined to anecdotal records (Brendonck et al., 1995; Wiman, 1981) and results of a lab experiment with the Sudanese fairy shrimp *Streptocephalus proboscideus* (Brendonck et al., 1995). These suggest sex-specific behaviour with conspicuous females being more benthic than males, probably to avoid predation by visual hunters such as birds and pelagic invertebrate predators. Here, we want to consolidate previous results and expand our knowledge on baseline behaviour in fairy shrimps. For this, we conduct a laboratory experiment to study how variation in spontaneous activity level and geotaxis (i.e., gravity-mediated activity) is determined by differences in sex and body length in the fairy shrimp *Branchipodopsis wolfi* Daday, 1910. Moreover, we assess how chronic exposure to an environmentally relevant concentration of the anxiolytic pharmaceutical fluoxetine – the active compound of Prozac – impacts behaviour, and if effects are sex- or size- dependant.

The studied species mainly inhabits shallow and clear temporary rock pools in southern and eastern Africa, and typically reaches sexual maturity in about six days after hatching (Pinceel et al., 2017a). Mature females tend to be larger than males, and have a conspicuous blue-red brood pouch. Based on this sexual dimorphism and on previous reports (Brendonck et al., 1995), we expect females to be less active and to spend more time near the bottom of the water column, probably as part of an antipredator strategy to reduce the probability of being detected by visual hunters. Because this risk typically increases with increasing body length, we expect that larger individuals will behave more cautiously than smaller ones. With respect to fluoxetine exposure, we hypothesize the compound to alleviate anxiety-related responses, resulting in activity closer to the surface of the water column. Fluoxetine is a selective serotonin reuptake inhibitor with anxiolytic properties that binds to evolutionary-conserved 5-HT transporters in neurons to inhibit the re-uptake of the neurotransmitter serotonin in the synaptic cleft (Gunnarsson et al., 2008; Mcdonald, 2017). There is increasing evidence from other taxa including fish and water fleas that exposure to the compound inhibits risk-averse behaviour at environmentally relevant levels (but see Sumpter et al., 2014). For instance, fluoxetine stimulated activity in eastern mosquitofish (*Gambusia holbrooki*) after 28 days of exposure (Martin et al., 2017), and full life-cycle exposure increased swimming acceleration in turquoise killifish (*Nothobranchius furzeri*) (Thoré et al., 2021c). Medaka (*Oryzias latipes*) exposed to 100 μg/L fluoxetine for 10 days spent more time at the top of the water column (Ansai et al., 2016),

and negative phototactic behaviour decreased in *Daphnia magna* already after 8 days of exposure at 1 ng/L fluoxetine (Rivetti et al., 2016). Based on a cross-species extrapolation, we hypothesise a similar impact on fairy shrimps.

2. Materials and methods

2.1. Fairy shrimp hatching and husbandry

A mixed dry sediment sample with *B. wolfi* resting eggs was collected from ten different rock pools on Korannaberg mountain (Eastern Free State, South Africa) and transported to the laboratory at KU Leuven. The sediment sample was inundated in 2 L plastic hatching trays (11 × 17 × 11.5 cm, approx. 50 mL sediment per tray) with oxygenated type II RO water at 20 °C. Seven days after inundation, hatchlings were transferred to 210 mL glass jars with reconstituted water (demineralized water with NaHCO$_3$, CaSO$_4$·2H$_2$O, MgSO$_4$ and KCL to 20 μS/cm conductivity, after Pinceel et al., 2017b) in which they were kept individually for the remainder of the experiment (Fig. 1). Organisms were reared under optimal common garden conditions in temperature-controlled incubators at 20 °C and a 12-h light: 12-h dark cycle (full spectrum white light, 4000 lx, lamp type Osram L 8 W/640; Osram, Rotterdam, the Netherlands) (cf. Pinceel et al., 2016). Water was renewed once per week to ensure optimal water quality. Individuals were fed *ad libitum* with live *Acutodesmus obliquus* (CCAP 276/3A) algae twice per week.

2.2. Experimental set-up

Seven days after inundation, individuals were randomly assigned to one of two treatments: a control ($n = 51$ males and 47 females) and a condition in which fairy shrimps were continuously exposed to 0.5 μg/L fluoxetine ($n = 48$ males and 56 females) until the end of the experiment. Fluoxetine is a relatively persistent compound with a > 100-days half-life in water (Kwon and Armbrust, 2006), and occurs in surface waters at concentrations < 0.6 μg/L (Puckowski et al., 2016; Saaristo et al., 2017). Three weeks after inundation, each individual was subjected to two different behavioural tests: an open-field test to assess spontaneous activity level and a diving test to measure activity with regard to depth in the water column (geotaxis) (Fig. 1). Both tests were performed between 12.00 am and 16.00 pm to minimize potential daily behavioural variation, and were separated from each other by 48 h. At the onset of each test, an individual was introduced to an experimental arena and allowed to acclimate for five minutes. Subsequently, behaviour was recorded for 10 min using Logitech C920 HD Pro Webcam digital cameras. Recordings were analysed (observer-blind) using Ethovision XT Ver. 14.0 video-tracking software (Noldus Information Technologies, Wageningen, the Netherlands) for open-field data. Diving test recordings were analysed manually. Individuals were visually separated from each other during these tests to avoid any confounding social interactions.

The open-field test ran in a barren test arena (11 × 17 × 11.5 cm) with a water level of 2 cm (0.5 L) to confine the test individual to primarily two-dimensional movement. The arena had a white base to ensure sufficient contrast between individual and background (dorsal view, Fig. 1), including a 2 × 2 cm grid for size-calibration. Travelled distance (in cm) during the 10-minutes observation period was calculated as a measure for spontaneous activity level. Moreover, a screenshot was obtained when the individual was centred in the frame to determine total body length using the open source image processing software ImageJ Version 1.50i (Schneider et al., 2012). Total body length was measured from the top of the head to the base of the tail (furca) (dorsal view).

The diving test ran in a narrow but high barren test arena (20 × 6 × 20 cm) with a water level of 16 cm to allow for sufficient vertical movement (front view, Fig. 1). A 2 × 2 cm grid on a white background was provided to virtually divide the water column in 4 horizontal layers
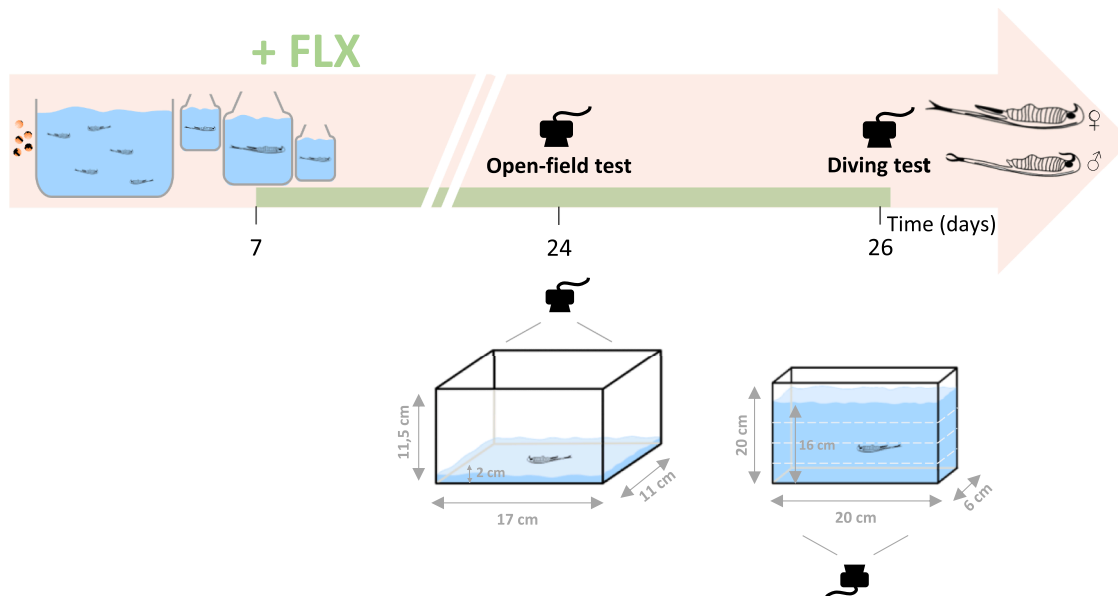
Fig. 1. Experimental setup. Starting 7 days after inundation, *B. wolfi* were housed individually in 210 mL jars and randomly assigned to either a control or a condition in which individuals were chronically exposed to 0.5 µg/L fluoxetine. At day 24 and 26, individuals were subjected to an open-field test and a diving test, respectively. In addition, total body length was assessed at day 24 during the open-field test.

(each zone with a height of 4 cm). The total number of times an individual entered a new layer during the 10-minutes observation period was assessed as a measure of vertical activity. In addition, the mean depth in the water column was assessed as a measure for risk-averse behaviour, i.e. benthic behaviour (or positive geotaxis) is considered more risk-averse than pelagic behaviour (or negative geotaxis). To this end, a mean height score was calculated for each individual as the cumulative time spent in each layer multiplied with a fixed score for that layer (score 1 for the bottom layer, score 4 for the surface layer), divided by the total observation time (600 s). Therefore, a higher score indicates that the individual on average resides closer to the surface, and vice versa.

2.3. Exposure medium

A 5 mg/L fluoxetine stock solution was prepared by dissolving fluoxetine hydrochloride (F-132, Sigma-Aldrich) in demineralised water, and preserved as 50 mL aliquots at −20 °C until use. Stock solution aliquots were thawed one hour before use and added to the jars of the fluoxetine condition to a nominal concentration of 0.5 µg/L (cf. protocol by Thoré et al., 2018). The treatment was applied once per week when the water in the jars was renewed. Throughout the experiment, mixed water samples were taken from three randomly selected jars (to a volume of 10 mL/sample) before renewal of the water, and analysed using liquid chromatography coupled to mass spectrometry (LC/MS/MS) to analyse for the presence of fluoxetine hydrochloride. The mean detected level of fluoxetine in the samples was 0.056 µg/L (STDEV: 0.025 µg/L, $n = 9$).

2.4. Statistical analysis

All statistical tests were performed in R 4.0.3 (R Development Core Team, 2016) assuming an alpha = 0.05 significance level. Model assumptions for all analyses were verified graphically.

Total body length was analysed by means of a linear model with a Gaussian error distribution, with sex (male, female) and treatment (control, fluoxetine) as well as their interactions as fixed predictors. Travelled distance (open-field test) and mean height score (diving test) were analysed by means of a linear model with a Gaussian error

distribution, with sex, treatment and body length, as well as their interactions, as fixed predictors. The mean height score was log-transformed to improve the distributional fit. The frequency by which individuals changed between horizontal layers was analysed using a similar model with Poisson error distribution.

3. Results

Female fairy shrimps (mean $\pm$ SE 10.8 $\pm$ 0.1 mm) were on average 8% larger than males (mean $\pm$ SE 10.0 $\pm$ 0.1 mm) ($F_{1,193} = 16.858$, $P < 0.001$). Fluoxetine treatment had no overall impact on body length ($F_{1,193} = 2.271$, $P = 0.133$), nor did fluoxetine treatment affect body length differentially in males and females ($F_{1,193} = 0.063$, $P = 0.802$).

Males travelled on average a 27% longer distance (mean $\pm$ SE 1113.890 $\pm$ 63.122 cm) during the open-field test than females (mean $\pm$ SE 873.767 $\pm$ 59.388 mm) (Fig. 2A, Table 1). Moreover, for both males and females, larger individuals travelled further than smaller individuals (Fig. 2B, Table 1). Fluoxetine did not affect the travelled distance (Table 1).

Males were on average 107% more vertically active during the diving test and changed more frequently (mean $\pm$ SE 21.928 $\pm$ 0.495 times) between depth zones than females (mean $\pm$ SE 10.570 $\pm$ 0.335 times). However, this effect depended on both body length and fluoxetine exposure (Table 1). While non-exposed females were less vertically active with increasing body length, the opposite relationship was found for non-exposed males (Fig. 2C). This relationship was far less pronounced or even absent when fairy shrimps were exposed to fluoxetine (Fig. 2C). Irrespective of body length and fluoxetine treatment, males resided on average 26% closer (height score: mean $\pm$ SE 1.681 $\pm$ 1.045) to the surface than females (height score: mean $\pm$ SE 1.336 $\pm$ 1.044) (Fig. 2D, Table 1).

4. Discussion

Neurochemicals have been shown to alter the behaviour of several commonly used freshwater model organisms in ecotoxicological assays. Still, it is difficult to generalise these results and make biologically meaningful predictions across taxa. This is partly due to a lack of studies on species with non-generic life-history strategies, such as many
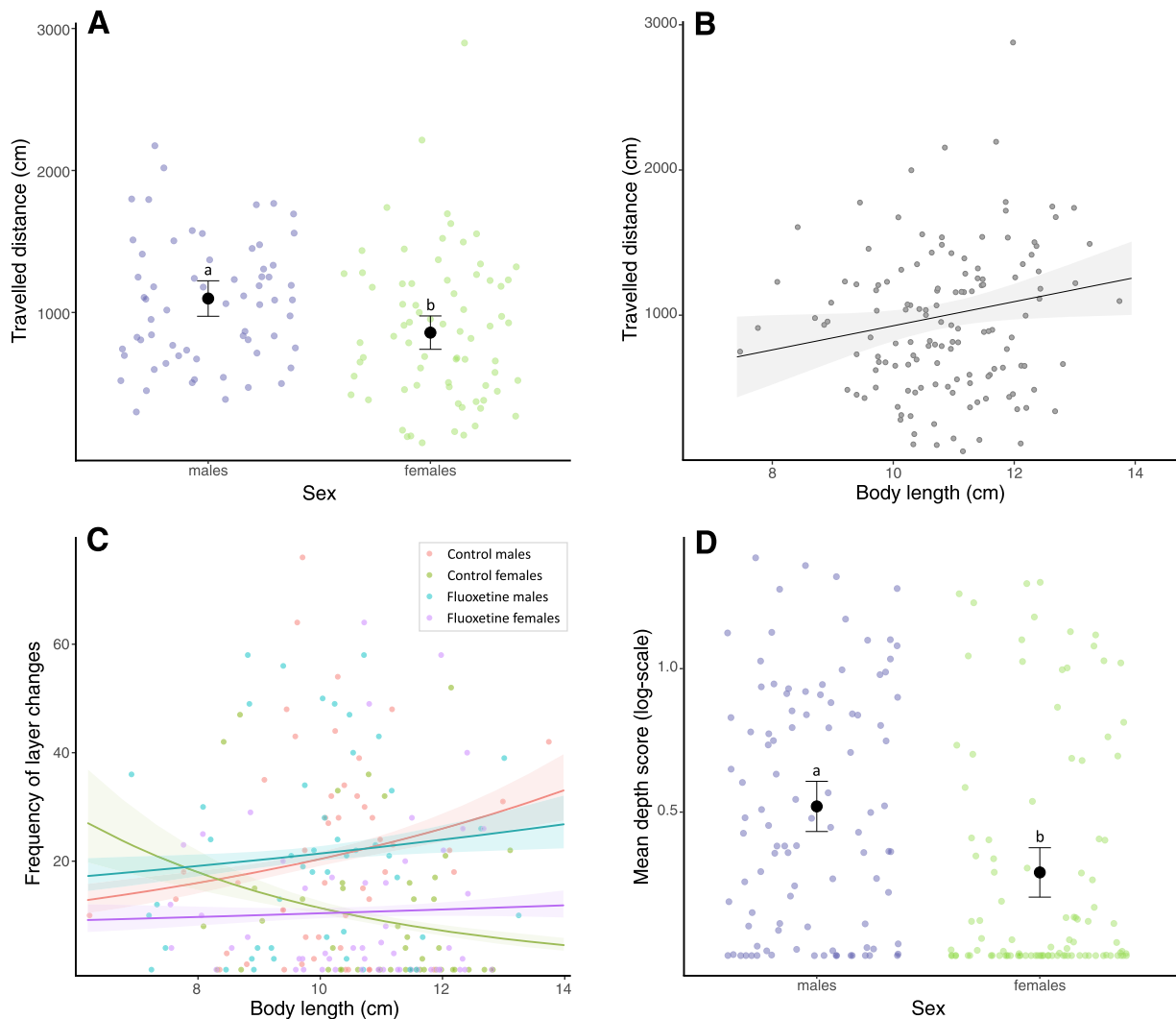
Fig. 2. *Branchipodopsis wolfi* behaviour in the open-field and diving test in relation to body length, sex and fluoxetine exposure. Travelled distance (cm) during a 10-minutes open-field test was assessed as measure for spontaneous activity level and depended on (A) sex and (B) body length. (C) Frequency of layer changes during a 10- minutes diving test was assessed as measure for vertical activity and depended on body length, sex and fluoxetine exposure, while (D) the mean height in the water column depended on sex. Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences.

Table 1
Linear regression model output for travelled distance by *B. wolfi* (open-field test), frequency of layer changes (diving test) and mean height score (diving test).

Effect	Travelled distance			Frequency of layer changes			Mean height score		
	F	P-value	df_1, df_2	χ^2	P-value	df_1, df_2	F	P-value	df_1, df_2
Sex	7.393	**0.007**	1, 128	391.050	**≤0.001**	1, 189	14.846	**≤0.001**	1, 189
Treatment	2.659	0.105	1, 128	1.010	0.314	1, 189	1.241	0.267	1, 189
Body length	4.391	**0.038**	1, 128	6.020	**0.014**	1, 189	0.437	0.509	1, 189
Sex*Treatment	0.053	0.818	1, 128	0.030	0.854	1, 189	0.895	0.345	1, 189
Sex*Body length	0.141	0.707	1, 128	32.990	**≤0.001**	1, 189	0.595	0.441	1, 189
Treatment*Body length	0.367	0.545	1, 128	2.860	0.091	1, 189	0.241	0.624	1, 189
Sex*Treatment*Body length	0.867	0.354	1, 128	33.060	**≤0.001**	1, 189	1.083	0.299	1, 189

P-values < 0.05 are shown in bold and underlined.

freshwater invertebrates, and a lack of sufficient baseline behavioural information. Here, we characterised baseline antipredator-related behaviour for a widespread fairy shrimp from temporary waters and show that exposure to fluoxetine, the active compound of Prozac, disrupts ecologically-relevant trade-offs between behaviour and morphology.

4.1. Baseline behaviour of freshwater fairy shrimps

Overall, larger fairy shrimps were more active than smaller individuals in our trials. While this finding suggests that biophysical differences underlie variation in activity level, also sex-specific patterns emerged. Although larger than males, females were less active than males. This result is in line with earlier observations that female *Streptocephalus* fairy shrimps swim at a lower speed compared to males

(Wiman, 1981) and could suggest that males and females experience different selection pressures with regard to behavioural expression. Because female *B. wolfi* are larger than males and have a conspicuously coloured brood pouch, they may be more susceptible to visual predation by birds and pelagic invertebrate hunters (Brendonck et al., 1995). Given that reduced activity is a common strategy to limit predation pressure in amphipods (Arnal et al., 2015), this likely underpins the observed patterns. Consistent with this hypothesis, our results show that female fairy shrimps are less vertically active and more benthic than males. Furthermore, larger females were less vertically active than smaller ones. In aquatic organisms, the natural tendency to dive to the bottom of the water column is often used as a measure for anxiety-related or risk-averse behaviour (Parker, 2016). Although it has not yet been confirmed whether this also holds for fairy shrimps, our results are at least suggestive of sex-specific differences in risk-averse behaviour.

Interestingly, larger males were more vertically active than smaller ones. Because males are less conspicuous than females, predation is likely less of a threat to males than females. Furthermore, in many freshwater crustaceans, males actively explore the water column to look for suitable mates and increase their reproductive success (Belk, 1991). Previous research suggests that larger fairy shrimp males perform more mating attempts than small ones (Feigenbaum and Switzer, 2007), and higher observed vertical activity of larger males in our trials may therefore reflect an increased mating intent. Moreover, pelagic activity may also decrease the predation risk by benthic flatworms (De Roeck et al., 2005) and reduce the probability of infection by sediment-associated parasites (Decaestecker et al., 2002). Although vertical migration in aquatic organisms is commonly interpreted in an antipredator context (Brierley, 2014; Wudkevich et al., 1997), the response may be confounded by a range of non-mutually exclusive adaptive drivers. For instance, individuals could visit deeper water to avoid photodamage due to high UV levels (Brendonck et al., 1995; Brierley, 2014) or to avoid extreme temperatures which can be common during daytime in temporary ponds of (semi-)arid regions (Williams, 2006). By complementing ecologically-relevant tests in the laboratory with *in situ* tests in the field, future research should further scrutinize the underlying motivation and fitness consequences of the observed responses.

4.2. Neurochemical exposure affects morphology-behaviour trade-offs

Serotonin is a prime neurotransmitter in many different organisms and is linked to various behavioural responses in crustaceans (Fong and Ford, 2014). For instance, altered serotonergic activity due to parasite infections was shown to disturb normal phototactic behaviour in both freshwater and marine amphipods (Guler and Ford, 2010; Tain et al., 2006). Similarly, CRISPR-Cas9 mutated *Daphnia magna* that lacked serotonin were more active and responsive to light than wild type daphnids (Rivetti et al., 2018). Because drug target molecules of antidepressants - selective serotonin reuptake inhibitors in specific - are often evolutionary conserved in crustaceans (Fong and Ford, 2014; Gunnarsson et al., 2008), it can reasonably be expected that exposure to these compounds disrupts crustacean neurophysiology and behaviour. Such effects are increasingly well documented for crayfish, a common model in neurophysiological research, and in amphipods and daphnids as traditional models in ecotoxicology (Fong and Ford, 2014). For instance, fluoxetine increased extracellular serotonin levels and resulted in bigger clutches of smaller offspring in *Daphnia magna* (Campos et al., 2016, 2012). Nevertheless, effects of exogenous neurological modulators cannot be readily extrapolated across species (Fong and Ford, 2014; Pasztor and Macmillan, 1990). This is especially true for species with non-generic life-histories and for which the behavioural baseline is poorly understood (Kohler et al., 2018a, 2018b). In case of aquatic organisms from temporary pools, for instance, it is expected that physiological adaptations to deal with the extensive daily and seasonal variation inherent to their habitat (Brendonck et al., 2017) may influence pollutant sensitivity (Lahr, 1997). Consistent with this notion, we found that chronic exposure to 0.5 μg/L fluoxetine did not affect activity level and average depth in the water column in the studied fairy shrimps, whereas previous studies did report such responses in water fleas (Nielsen and Roslev, 2018) and amphipods (Guler and Ford, 2010) after exposure to similar concentrations of the compound. Another study showed that 8-day exposure to fluoxetine already impacted negative phototactic behaviour in water fleas at concentrations as low as 1 ng/L (Rivetti et al., 2016). Despite potential species-specific responses, it is noteworthy that non-monotonic concentration-dependant responses are often observed (Fong and Ford, 2014). For instance, female mosquito-fish displayed less anxiety-related behaviour at 61 ng/L but not 352 ng/L fluoxetine, while males were more anxious at 352 ng/L but not 61 ng/L fluoxetine (Martin et al., 2019). Hence, ideally, a broader range of environmentally relevant concentrations should be tested to compare the sensitivity of species to the compound (Harris et al., 2014). Furthermore, results should ideally be shown repeatable before differential outcomes of studies can be meaningfully compared (Harris et al., 2014; Sumpter et al., 2014).

Our findings are only a first contribution to compiling a behavioural baseline for fairy shrimps and to assessing the impact of neurochemical exposure. Consequently, many open research questions remain. For instance, in a previous study, diurnal migratory patterns were suggested for fairy shrimps (Brendonck et al., 1995). Whether these are repeatable and structure behavioural responses remains to be studied. Such patterns may not only confound the results of ecotoxicological testing but can also be developed into sensitive endpoints. For instance, a recent study showed that the behavioural impact of antidepressant exposure in fish depends on the time of day, and that daily activity changes disappear upon exposure (Thoré et al., 2021a). Behaviour is not only naturally variable, but it also tends to co-vary with life-history or morphological traits in a sex-dependant way (Hämäläinen et al., 2018; Thoré et al., 2019). Yet, many laboratory studies including behavioural trials in ecotoxicology do not fully consider the potential confounding impact of these trade-offs and differential responses (Cherry et al., 2020; Thoré et al., 2021a). Although fluoxetine exposure had no overall impact on activity level and geotaxis in the studied crustaceans, it did disrupt the sex-specific trade-off between body length and vertical activity, something which was only revealed because both were included in the analysis. Because this trade-off likely evolved in response to several selection pressures including predation and sexual selection, exposure to fluoxetine is likely to affect reproductive success and survival. These findings not only contribute to an understanding of the adaptive value of fairy shrimp behaviour, but also illustrate how neurochemical pollution may disrupt important phenotypic trade-offs in freshwater organisms.

CRediT authorship contribution statement

Eli.S.J. Thoré: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Luc Brendonck:** Supervision, Writing – review & editing. **Tom Pinceel:** Conceptualization, Writing – original draft.

Declaration of Competing Interest

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

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