Aquatic Toxicology 232 (2021) 105743

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Generation-specific and interactive effects of pesticide and antidepressant exposure in a fish model call for multi-stressor and multigenerational testing

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ARTICLE INFO

Keywords:
Nothobranchius
Emerging pollutants
Fluoxetine
3,4-dichloroaniline
Behavior
Pollutant mixture

ABSTRACT

Ecological risks of a pollutant are typically assessed via short-term exposure of model organisms to that single compound. Such tests are informative, but cannot ascertain effects of long-term and multigenerational mixed-stressor exposure with which organisms are often confronted in their natural environment. Therefore, full life-cycle and multigenerational tests are needed. Yet, these are hampered due to long lifespans and generation times of many standard laboratory species, in particular for vertebrates such as fish. With a typical lifespan of 6 months and a generation time of about 3 months, the turquoise killifish (*Nothobranchius furzeri*) may be an ideal model for multigenerational testing. In this study, we assessed the impact of full life-cycle exposure to the emerging pollutant fluoxetine (0, 0.5 µg/L) in combination with chronic exposure during adulthood to the pesticide 3,4-dichloroaniline (0, 50, 100 µg/L) over two successive generations of *N. furzeri*. Overall, both life-history and behaviour were affected by exposure to fluoxetine and 3,4-DCA. Inhibitory effects of single chemical exposure on growth and fecundity were generation-dependent, while enhanced swimming acceleration and feeding in response to fluoxetine were dependent on the presence of 3,4-DCA. Together, these findings show the relevance of a multi-stressor approach across successive generations. Although full life-cycle and multigenerational tests are typically assumed to be impractical and costly for fish, we deliver an effective demonstration that such studies are possible within a timespan of less than 6 months with the killifish *N. furzeri* as a model organism.

1. Introduction

Natural ecosystems are increasingly polluted (Schwarzenbach et al., 2006). Next to conventional contaminants such as pesticides and heavy metals, emerging contaminants such as pharmaceutical compounds and personal care products could also threaten the environment (Santos et al., 2010; Li, 2014; Sauvé and Desrosiers., 2014). In aquatic environments, pharmaceuticals generally occur at relatively low concentrations (ng/L-µg/L) compared to conventional contaminants (µg/L-mg/L) (Arnold et al., 2014; Philippe et al., 2019). Still, this is worrying since pharmaceuticals are designed to be highly potent and to exert specific biological effects at very low concentrations (Arnold et al., 2014). Since drug target molecules are often evolutionary conserved across vertebrate phyla, fish are likely to be affected by pharmaceutical pollution (Gunnarsson et al., 2008). This is especially true for neuroactive compounds, which are assumed to be among the most ecotoxic drugs (Puckowski et al., 2016). Ecotoxicologists are now left with the challenge to identify which pharmaceutical compounds pose a threat, at what environmental concentrations, and what these threats entail (Tanoue et al., 2019).

To assess the environmental risks of pollutants, a battery of standard ecotoxicological tests is typically performed (cf. OECD guidelines for the testing of chemicals, Organisation for Economic Co-operation and Development) (Brodin et al., 2014; Klaminder et al., 2014). Examples of such tests include the fish acute toxicity test (OECD Test No. 203) and the early life-stage toxicity test (OECD Test No. 210). Although they

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https://doi.org/10.1016/j.aquatox.2021.105743
Received 16 July 2020; Received in revised form 22 December 2020; Accepted 4 January 2021
Available online 8 January 2021

rapidly deliver information for regulatory decision making, the ecological relevance of these standard tests may be low because they fail to ascertain 1) interactive effects between stressors that occur together in the environment, 2) specific biological effects (e.g., behavioural changes) that may not immediately and overtly affect organisms, and 3) potential effects of long-term exposure to persistent stressors.

First, organismal effects of each pollutant are tested in isolation whereas, in the natural environment, organisms are exposed to a mixture of stressors that can have additive, antagonistic or synergistic interactions (Darling and Côté., 2008; Galic et al., 2018). Since such interactions are largely ignored in standard ecotoxicity tests, the impact of pollution on aquatic organisms is often under- or overestimated (Liess et al., 2016; Philippe et al., 2019).

Second, most current ecotoxicological tests mainly focus on apical endpoints (indicating lethal or stressful effects) by assessing survival, reproduction and development (Klaminder et al., 2014; Thoré et al., 2018a). Nevertheless, pharmaceuticals may induce more subtle changes including behavioural and physiological alterations with severe fitness consequences (Brodin et al., 2013, 2014), but such effects are generally not assessed (Arnold et al., 2014; Klaminder et al., 2014). Consequently, traditional ecotoxicological tests may not consider pharmaceuticals as an environmental threat at current concentrations.

Third, current standard ecotoxicity screening generally focuses on acute or short-term exposure to pollutants, in which effects of long-term or multigenerational exposure are neglected (Philippe et al., 2017; Thoré et al., 2018a). Acute tests, however, do not always reflect a realistic exposure regime (Santos et al., 2010; Thoré et al., 2018a). For instance, pharmaceutical compounds generally occur in the environment at very low concentrations that are unlikely to exert effects after short-term exposure (Fent et al., 2006; Thoré et al., 2018a). Combined with the fact that many pharmaceuticals are highly persistent due to a low bio-degradability and are continuously discharged, organisms are often chronically exposed over long periods (Fent et al., 2006; Kwon and Armbrust, 2006; Arnold et al., 2014).

In this study, we assessed the feasibility and relevance of accommodating these shortcomings in ecotoxicological testing. To this end, we examined life-history and behavioural consequences of full life-cycle exposure to the emerging pollutant fluoxetine in combination with chronic exposure to the pesticide 3,4-dichloroaniline (3,4-DCA) across two successive generations of the fish model *Nothobranchius furzeri* (turquoise killifish). Full life-cycle and multigenerational exposure studies with traditional fish models (e.g., zebrafish *Danio rerio*) are deemed time consuming and expensive due to their long generation time and lifespan (Ankley and Villeneuve., 2006; Harel et al., 2015; Philippe et al., 2017). For instance, compared to zebrafish with a lifespan of ~ 5 year and a maturation time of ~ 12 weeks after hatching, *N. furzeri* has a shorter lifespan and a faster maturation of ~ 5–6 months and 3 weeks, respectively (Blažek et al., 2013; Cellerino et al., 2015). The species originates from temporary ponds in south-east Africa and deals with the transient nature of its habitat by maturing fast and producing drought-resistant eggs that remain dormant in the sediment during the dry season (Pinceel et al., 2015; Polačik et al., 2016; Grégoir et al., 2017). After an initial screening of the congeneric *N. rachovii* (van der Hoeven et al., 1982) and *N. guentheri* (Shedd et al., 1999), *N. furzeri* was recently selected and launched as a model organism for acute and chronic ecotoxicological tests (Philippe et al., 2017, 2018a,2018b, 2018c, 2019), as well as for behavioural testing (Thoré et al., 2018a, 2018b, 2019, 2020). Because of its fast pace-of-life, the species is promising as a time- and cost efficient model in any type of full life-cycle and multigenerational studies (Harel et al., 2015; Thoré et al., 2018a, 2018b).

We focused on the combination of an emerging and a conventional pollutant: fluoxetine is a selective serotonin-reuptake inhibitor (SSRI) with antidepressant and anxiolytic properties (Ansai et al., 2016), and the pesticide 3,4-DCA causes tissue hypoxia by reducing the oxygen binding affinity of haemoglobin (Crossland, 1990). Effects of both pollutants have been tested separately in killifish within one generation (Philippe et al., 2018b, 2019; Thoré et al., 2018a), but it is unknown how they may interact and affect organisms across successive generations. We assessed a combination of typical life-history (total body length, fecundity and fertilisation effectiveness) and behavioural traits with direct ecological relevance (open-field activity, anxiety-related behaviour and feeding behaviour). In addition, by adopting an individual-level repeated measures design, we were able to assess behavioural variability within and between individuals to calculate behavioural repeatability, which is the fraction of behavioural variation that is due to differences between individuals of the studied traits (Bell and Sih, 2007; Thoré et al., 2018b). Recent studies argue that the degree of behavioural variability and the baseline behaviour of model organisms need to be characterised (Harris et al., 2014; Tanoue et al., 2019). Because such information is often not generated by ecotoxicological studies, the exact impact and environmental risk of contaminants is usually not understood (Thoré et al., 2018b; Tanoue et al., 2019).

We hypothesised that chronic fluoxetine-exposure would inhibit feeding behaviour and, consequently, growth. Although the underlying mechanisms remain unknown, this negative effect of fluoxetine has consistently been shown in fish (McDonald, 2016). Furthermore, we expected an increased reproductive output in response to fluoxetine as recent studies revealed that fluoxetine-exposure stimulates reproduction in fish. For instance, male mosquitofish (*Gambusia holbrooki*) exposed to environmentally-relevant doses of fluoxetine for 35 days spent more time pursuing females and performed more mating attempts (Martin et al., 2019). Similarly, fluoxetine-exposed guppies engaged more in coercive sneak copulation (Fursdon et al., 2019). Given the anxiolytic properties of fluoxetine (Ansai et al., 2016), we also expected fish to display more risk-prone behaviour (i.e., more active, less anxiety-related behaviour) when exposed to fluoxetine. In terms of pesticide effects, we expected 3,4-DCA-exposed fish to exhibit a decreased growth, as previously shown in rainbow trout (*Oncorhynchus mykiss*) (Crossland, 1990) and fathead minnow (*Pimephales promelas*) (Call et al., 1987), that would likely be associated with impaired feeding behaviour. Furthermore, we expected a lowered reproductive output, activity and more anxiety-related behaviour upon exposure to 3,4-DCA. In support of this hypothesis, locomotor activity in early life stages of zebrafish was impaired upon exposure to 3,4-DCA (Scheil et al., 2009). As fluoxetine and 3,4-DCA were both expected to affect life-history and behaviour of *N. furzeri*, interactive effects between the two compounds were likely to occur. Finally, it has been shown that fluoxetine can inhibit the expression of behaviour-regulating genes (Robison et al., 2014). If these effects carry over to future generations, parental exposure to fluoxetine is likely to affect offspring (Parker, 2016; Thoré et al., 2018a). Consequently, we expected the effects of fluoxetine exposure to be equally strong or even more pronounced in the second generation of exposed fish.

2. Material and methods

2.1. Fish maintenance

Fish originated from a natural population in central Mozambique (strain MZCS-222), which was reared under optimal laboratory conditions (according to the protocol of Polačik et al. (2016)) for a minimum of three generations before the start of the experiment. To start the experiment, 'ready-to-hatch' eggs (stage 43 sensu Wourms, 1972) were inundated (protocol: Polačik et al. (2016)), with reconstituted water at a conductivity of 600 µS/cm (Instant Ocean salt mix added to type II RO water) enriched with 1 g/L humic acid (53680; Sigma-Aldrich) to stimulate hatching. Throughout the experiment, fish were kept at a 14h: 10h light: dark regime. Tanks were placed in a temperature-controlled water-bath system to ensure a constant water temperature of 24 °C (mean 24.2 ± SD 0.1 °C). Nine days post hatching (dph), larvae were transferred from hatching trays to 10L-tanks (49.5cm × 20cm × 18 cm)

Table 1
Sample size per sex per experimental group.

Experimental group	Treatment	Sex	Sample size	
			Generation 1	Generation 2
CTR	/	M	16	11
CTR	/	F	16	11
CD50	50 µg/L 3,4-DCA	M	18	7
CD50	50 µg/L 3,4-DCA	F	18	7
CD100	100 µg/L 3,4-DCA	M	15	7
CD100	100 µg/L 3,4-DCA	F	15	7
FLX	0.5 µg/L fluoxetine	M	17	11
FLX	0.5 µg/L fluoxetine	F	17	11
FD50	0.5 µg/L fluoxetine + 50 µg/L 3,4-DCA	M	16	4
FD50	0.5 µg/L fluoxetine + 50 µg/L 3,4-DCA	F	16	4
FD100	0.5 µg/L fluoxetine + 100 µg/L 3,4-DCA	M	18	1
FD100	0.5 µg/L fluoxetine + 100 µg/L 3,4-DCA	F	18	1
Total:			200	82

in social groups (mixed-sex) of five to six individuals per tank. Tanks were filled with aerated reconstituted water at 600 µS/cm conductivity (without addition of humic acid). Starting from nine dph and until 51 dph, water in the tanks was renewed once a week (every Monday). After each renewal event, fish were randomly re-distributed among replicate tanks of their respective experimental condition (see below). From 51 dph and until the end of the experiment (89 dph), fish were housed individually in 2L-jars to allow for individual monitoring. Water in the jars was renewed twice a week to ensure constant water quality conditions (every Tuesday and Friday; 7.8 pH, ammonium <0.2 mg/L, nitrite <25 mg/L). Juvenile fish were fed to satiation with live *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium) twice a day and additionally fed frozen *Chironomus* larvae (Ocean Nutrition, Essen, Belgium) from 22 dph onwards. From 51 dph onwards, fish were fed to satiation with *Chironomus* larvae once a day. Excess food was removed on a daily basis with a pipet to maintain good water quality.

2.2. Preparation of fluoxetine and 3,4-DCA solutions

A 5 mg/L fluoxetine stock solution was prepared by dissolving fluoxetine hydrochloride (Sigma F-132) in reconstituted water (600 µS/cm). This stock was stored as 40 mL aliquots at −20 °C until use. Stock solution was added to the tanks to a concentration of 0.5 µg/L for all fluoxetine units (FLX, FD50, FD100; Table 1). A 25 mg/L 3,4-DCA stock solution was prepared by adding 3,4-DCA (437778; Sigma-Aldrich) to reconstituted water. This stock solution was always prepared one day before fish water renewal and stirred overnight. 3,4-DCA stock solution was added to the tanks to a concentration of 50 µg/L (CD50, FD50; Table 1) or 100 µg/L (CD100, FD100; Table 1) (cf. Philippe et al., 2019). Following this protocol, actual concentrations in the medium were 30 % (SD = 0.04 µg/L) of the intended concentration for fluoxetine (total n = 30), and 77 % (SD = 2.12 µg/L) and 73 % (SD = 16.26 µg/L) of the intended high and low concentration of 3,4-DCA, respectively (total n = 4 mixed water samples). Compound concentrations were quantified by means of liquid chromatography (LC/MS/MS) with ESI (Waters ACQUITY UPLC, Xevo TQD mass spectrometer).

2.3. Experimental setup

Using a multigenerational design, fish were exposed to pollutants across two successive generations. Eggs produced by the first experimental generation were retained on peat, separate per experimental condition, and incubated at a constant temperature of 28 °C in constant light conditions (protocol: Philippe et al., 2018c). These eggs were used to hatch the second experimental generation. A total of 200 and 82 experimental fish were hatched in the first and second generation, respectively (Table 1).

Nine dph, fish were randomly assigned to one of two experimental conditions (in groups of 5–6 fish per tank, see above): a control condition and a condition in which fish were exposed to 0.5 µg/L fluoxetine until the end of the experiment. At 51 dph, fish were exposed to 3,4-DCA until the end of the experiment (individually in jars, see above). To this end, both conditions were divided in three sub-groups to a total of six experimental conditions. The control condition was divided into a control group (CTR), a group exposed to 50 µg/L 3,4-DCA (CD50) and a group exposed to 100 µg/L 3,4-DCA (CD100). Similarly, the fluoxetine condition was divided into a group that did not receive 3,4-DCA treatment (FLX), a group exposed to 50 µg/L 3,4-DCA (FD50) and a group exposed to 100 µg/L 3,4-DCA (FD100). Each experimental condition had an equal amount of males and females (Table 1). A full life-cycle and a shorter chronic exposure at environmentally relevant concentrations was chosen for fluoxetine and 3,4-DCA, respectively, to reflect a possible exposure scenario in a natural setting with exposure over long time periods for (pseudo-)persistent pharmaceuticals and seasonality of pesticide application. Fluoxetine is relatively resistant to degradation with a half-life that exceeds 100 days in aqueous solutions (Kwon and Armbrust, 2006), and occurs in surface waters at concentrations < 600 ng/L (Puckowski et al., 2016; Saaristo et al., 2017). 3,4-DCA has been detected in the environment at concentrations up to 567 µg/L (Primel et al., 2007; Philippe et al., 2019).

Starting from 59 dph (i.e., one week after initiating the 3,4-DCA-exposure), fecundity and behavioural data were collected once a week and for a total of four repeated measures per individual (i.e., over four consecutive weeks).

Every Monday, fish were allowed to spawn for two hours. To this end, fish of the same treatment group were transferred in random male-female pairs (each time a different partner) to 1L-jars with sand as spawning substrate (protocol: Philippe et al., 2018c). After two hours of spawning, fish were transferred back to their respective housing jars. Sand was sieved and the number of deposited eggs was counted as a measure for female fecundity. After each spawning session, the proportion of (un)fertilised eggs per experimental condition was assessed as a measure for fertilisation effectiveness.

Fish were divided in two cohorts to improve the feasibility of behavioural data collection. To assess open-field activity level and anxiety-related behaviour (measured as thigmotaxis: the tendency to remain close to the walls), fish were individually subjected to an open-field test on Wednesday and Thursday morning for cohort 1 and 2, respectively. For this, fish were individually transferred to a barren open-field test arena (17.4cm × 11.2cm × 11.5 cm) with a water level of 2 cm (0.5 L) to allow horizontal movement while limiting vertical movement. Fish were allowed to acclimate for five minutes after which their activity was recorded (top-view) for 15 min. As a measure of open-field activity, the total distance moved and the total amount of time during which the fish was moving were assessed. In addition, to allow for an assessment of anxiety-related behaviour, the open-field arena was virtually divided in a centre (50 % of arena length and width) and peripheral zone. Activity in the centre zone of the arena is considered to be risk-prone behaviour, while activity in the peripheral zone is considered to be risk-averse behaviour (Ansai et al., 2016). Cumulative time spent in the centre of the arena and the mean distance of the fish relative to the arena centre were assessed as measures for fish anxiety-related behaviour. After the test, fish were transferred back to their respective housing jars.

To assess feeding behaviour, fish were individually subjected to a feeding test (on Wednesday and Thursday afternoon for cohort 2 and 1, respectively). To this end, fish were individually transferred to a barren test arena (17.4cm × 11.2cm × 11.5 cm) with a water level of 2 cm (0.5 L). Fish were allowed to acclimate for five minutes, after which frozen *Chironomus* larvae were added to the arena as food. Subsequently, fish

Table 2
Model output for life-history variables.

	Total length at day 51		Total length at day 88		Number of produced eggs		Fertilisation effectiveness	
Effect	*F*	*P*-value	*F*	*P*-value	χ^2	*P*-value	χ^2	*P*-value
Sex	111.633	**<0.001**	254.727	**<0.001**				
Fluoxetine	11.412	**0.001**	0.275	0.600	2.526	0.112	0.399	0.527
Pesticide			1.597	0.204	11.486	**0.003**	3.078	0.215
Generation	37.529	**<0.001**	25.762	**<0.001**	23.470	**<0.001**	0.037	0.847
Cohort			3.703	0.055	5.215	**0.022**		
Total length					1.817	0.178		
Sex:Fluoxetine	0.778	0.378	1.136	0.287				
Sex:Pesticide			2.666	0.071				
Fluoxetine:Pesticide			2.341	0.098	0.404	0.847	5.723	0.057
Sex:Generation	16.353	**<0.001**	31.361	**<0.001**				
Fluoxetine:Generation	4.878	**0.028**	1.989	0.160	10.014	**0.001**	0.011	0.916
Pesticide:Generation			1.791	0.169	9.320	**0.009**	1.016	0.602
Sex:Fluoxetine:Pesticide			0.996	0.371				
Sex:Fluoxetine:Generation	1.346	0.247	2.518	0.114				
Sex:Pesticide:Generation			1.525	0.220				
Fluoxetine:Pesticide:Generation			1.262	0.285	2.660	0.264	6.356	**0.042**
Sex:Fluoxetine:Pesticide:Generation			0.661	0.517				

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

feeding behaviour was recorded (top-view) for 15 min. As a measure of feeding behaviour, latency time to initiate feeding upon food administration was assessed. Fish were abstained from food for 24 h before the feeding test to prevent disinterest in the provisioned food. Fish that did not start feeding within 15 min were given the maximum score of 900 s. After the test, fish were transferred back to their respective housing jars.

To increase the contrast between the focal fish and the background, all test arenas had a white base. In addition, to exclude the confounding effect of direct social interaction, fish were unable to see each other during the tests. Logitech C920 HD Pro Webcam digital cameras were used to record fish behaviour. Recordings were analysed afterwards (observer-blind) using Ethovision XT Ver 14.0 video-tracking software (Noldus Information Technologies) for open-field data. Recordings of feeding behaviour were analysed manually.

Individual total length (from the tip of the snout to the tip of the tail, dorsal view) was assessed twice over the course of the experiment, on day 51 (i.e., at the onset of 3,4-DCA-exposure) and on day 88 (i.e., at the end of the experiment). To measure total length, individual fish were briefly transferred to a Petri dish with a small amount of water upon which size-calibrated photographs were taken (Samsung Galaxy S8+ dual-pixel 12.0 M P AF F/1.7 camera, with fish centred in the camera frame to ensure correct measurement). Photographs were analysed afterwards using the open source image processing software ImageJ Ver 1.50i (Schneider et al., 2012).

2.4. Animal welfare note

Procedures and methods were in accordance with the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P070/2016). Two researchers (E. S. J. Thoré and F. Van Hooreweghe) independently checked the condition and health of each individual fish at least twice a day. All fish were housed under optimal conditions and water quality was monitored daily (7.8 pH, ammonium <0.2 mg/L, nitrite <25 mg/L). Disturbance and handling was kept to a minimum to prevent and limit stress.

2.5. Statistical analyses

All statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2016) at a significance level of alpha = 0.05. Model assumptions, including distributional fit and homogeneity of variances, were verified graphically for all analyses.

Total length (mm) at 51 dph was analysed using a linear model with Gaussian error distribution. Sex (male, female), fluoxetine treatment (control, 0.5 µg/L fluoxetine) and generation (generation 1, generation 2), including their interaction, were modelled as fixed factors. Likewise, total length (mm) at day 88 was analysed using a linear model with Gaussian error distribution. Sex, fluoxetine treatment, pesticide treatment (control, 50 µg/L 3,4-DCA, 100 µg/L 3,4-DCA) and generation were modelled as fixed factors, including full interactions. Cohort (cohort 1, cohort 2) was added as an additional fixed factor. Total distance moved (cm), total time moving (sec), mean velocity (cm/sec) and maximum acceleration (cm/sec^2) as measures for open-field activity were analysed with linear mixed effect models (lme4 package; Bates et al., 2017) with sex, fluoxetine treatment, pesticide treatment and generation as fixed factors, including their full interaction. In addition, total length (at 51 dph) was added as covariate and cohort as an additional fixed factor. Fish identity and trial number (referring to the repeated measures) were added as random effects. A Gaussian error distribution was assumed for all these models. Similar models were used to analyse measures of anxiety-related behaviour in the open field test, including cumulative duration in the centre (sec) and mean distance to the centre (cm). Likewise, latency time to initiate feeding (sec) in the feeding test was analysed using a similar model. To improve distributional fit, mean velocity, maximum acceleration and latency time to initiate feeding were log-transformed. Cumulative time spent in the centre was log +1-transformed. Female fecundity was measured as the number of produced eggs and was analysed using a linear mixed effect model with Poisson error distribution. Fluoxetine treatment, pesticide treatment and generation, including their full interaction, were modelled as fixed factors. Total length (on day 51) was added as covariate and cohort as an additional fixed factor. Fish identity and trial number were added as random effects. In addition, an observation-level random effect was modelled to accommodate overdispersion. A linear mixed model with binomial error distribution was used to analyse the fraction of fertilised eggs over the total egg count as measure for fertilisation effectiveness. Fluoxetine treatment, pesticide treatment and generation (including full interaction) were modelled as fixed factors, while trial number was added as random effect. In addition, to accommodate overdispersion, an observation-level random effect was modelled.

Significance of the fixed effects in the mixed models was tested by means of parametric bootstrapping with 1000 simulations using the afex package (Singmann et al., 2017). Post-hoc differences were assessed by means of Tukey-corrected pairwise comparisons using the lsmeans package (Lenth & Love, 2017). Although Tukey-corrected pairwise comparisons are used to control the probability of making type I errors

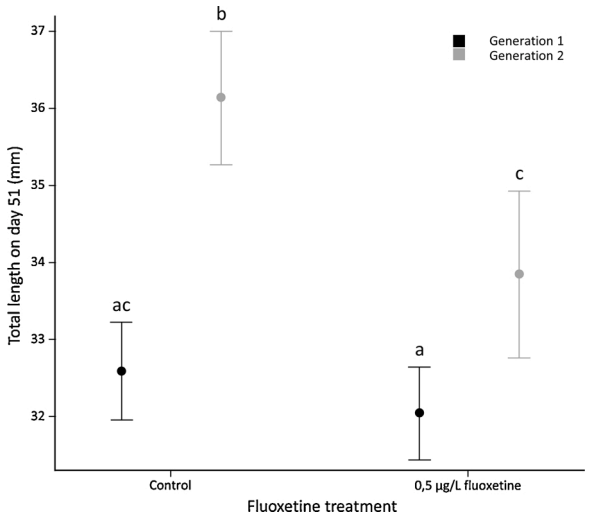

Fig. 1. Impact of fluoxetine-exposure on total body length at 51 dph (predicted effects). Letters indicate significant differences based on Tukey-corrected post-hoc tests. Whiskers delineate the upper and lower 95 % confidence limit.

within each test, there is still a 5% chance of type I errors among tests that should be taken into consideration upon interpretation of the results.

To determine if individual variation in the above behavioural traits was repeatable, repeatability measures were retrieved using the rptR package (Stoffel et al., 2018). Repeatability was calculated as the between-individual variance over the sum of between-individual and residual variance (Nakagawa & Schielzeth, 2010). To test the statistical significance of the repeatability values, likelihood-ratio tests (comparing the model with and without the fish identity random effect) were performed in the rptR package.

3. Results

3.1. Life-history

Adult total body length of males at 51 dph was significantly greater than that of females (Table 2, Fig. A1). Moreover, males of the second generation (37.8 mm ± 0.491 SE) were longer compared to males of the first generation (33.6 mm ± 0.3 SE). Female fish had an average body size of 31.1 mm ± 0.3 SE 51 dph. The same pattern was observed for body size at 88 dph as males of the second generation (46.1 mm ± 0.6 SE) were longer compared to males of the first generation (40.9 mm ± 0.3 SE). Female fish had an average length of 35.9 mm ± 0.3 SE at 88 dph (Fig. A1). Fluoxetine-exposed fish were smaller compared to control fish at 51 dph (Table 2). However, this effect was only apparent in the second generation of exposure, during which control fish had an average length of 36.1 mm ± 0.4 SE compared to fluoxetine-exposed fish which were 33.8 mm ± 0.5 SE (Fig. 1). Neither fluoxetine- nor pesticide treatment affected the length of adults at 88 dph (Table 2).

Fecundity was significantly reduced upon exposure to fluoxetine, although this effect only emerged in the first generation of exposure (Table 2, Fig. 2A). Similarly, exposure to 3,4-DCA decreased fecundity in the first but not in the second generation (Table 2, Fig. 2B). Overall, egg production was significantly higher in the second compared to the first generation (Table 2). The proportion of fertilised eggs over the total number of produced eggs as a measure of fertilisation effectiveness did not differ between generations and experimental conditions (Table 2).

3.2. Open-field activity

Total distance moved and total time moving in the open field as measures for open-field activity did not differ between sexes or generations (Table 3). Pesticide-exposure did not affect total distance moved. Fluoxetine-exposed fish of the second generation swam a significantly larger distance compared to control fish, whereas this effect was not apparent in the first generation (Table 3). Similarly, fluoxetine did not affect total time moving of first-generation fish but total time moving in the second generation was significantly affected by fluoxetine-exposure. The generation-specific effect of fluoxetine-exposure was dependent on pesticide-treatment (Table 3). Post-hoc analysis showed that the impact of fluoxetine on total time moving of second-generation fish only emerged upon exposure to low levels of 3,4-DCA, with fish exposed to fluoxetine spending more time moving compared to control fish (Tukey $P = \mathbf{0.001}$; Fig. 3A).

Mean swimming velocity did not differ between sexes or generations and was not affected by pesticide exposure (Table 3). Results suggest a generation-specific effect of fluoxetine. While there was no impact of fluoxetine-exposure on mean velocity in the first generation, exposed fish in the second generation swam at a significantly higher mean velocity than control fish (Fig. A2). This result was, however, not confirmed by post-hoc analysis. Fish total body length was linked to maximum acceleration, with bigger fish having a higher maximum

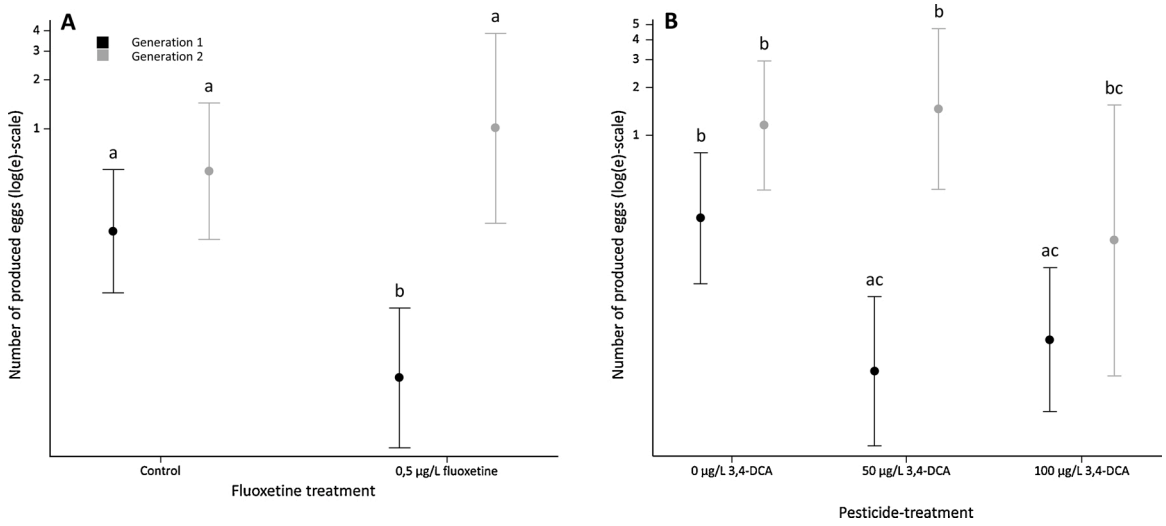

Fig. 2. Impact of pollutant-exposure on fish fecundity (predicted effects). The effect of (A) fluoxetine-exposure and (B) pesticide-exposure on the number of produced eggs per generation. Letters indicate significant differences based on Tukey-corrected post-hoc tests. Whiskers delineate the upper and lower 95 % confidence limit.

Table 3
Model output of variables related to fish open-field activity.

	Distance travelled		Total time moving		Mean Velocity		Maximum acceleration	
Effect	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value
Sex	−0.035	1.000	0.016	0.907	−0.067	1.000	15.161	**0.001**
Fluoxetine	1.727	0.200	3.662	0.077	0.705	0.410	2.337	0.141
Pesticide	2.347	0.352	3.823	0.184	2.039	0.397	1.363	0.556
Generation	2.446	0.164	1.881	0.190	3.359	0.086	7.620	**0.006**
Cohort	2.449	0.316	3.287	0.080	4.184	0.052	0.597	0.442
Total length	2.839	0.111	2.314	0.158	3.258	0.097	37.974	**0.001**
Sex:Fluoxetine	−0.041	1.000	−0.068	1.000	0.166	0.715	1.164	0.323
Sex:Pesticide	3.901	0.172	5.122	0.088	4.379	0.144	2.393	0.337
Fluoxetine:Pesticide	3.624	0.223	4.564	0.111	2.614	0.306	11.337	**0.006**
Sex:Generation	3.778	0.054	4.053	0.056	3.475	0.080	0.616	0.476
Fluoxetine:Generation	4.533	**0.045**	7.556	**0.010**	4.312	**0.048**	1.450	0.249
Pesticide:Generation	0.243	0.889	0.183	0.916	0.497	0.808	19.154	**0.002**
Sex:Fluoxetine:Pesticide	0.077	0.965	0.034	0.987	0.494	0.799	1.555	0.487
Sex:Fluoxetine:Generation	0.041	0.842	0.202	0.681	0.050	0.831	2.719	0.114
Sex:Pesticide:Generation	2.221	0.366	3.187	0.239	2.240	0.362	0.595	0.771
Fluoxetine:Pesticide:Generation	5.831	0.074	9.137	**0.015**	5.116	0.114	2.429	0.339
Sex:Fluoxetine:Pesticide:Generation	2.952	0.253	4.445	0.126	2.037	0.410	0.003	1.000

P-values < 0.05 are shown in bold.

acceleration compared to smaller fish (Table 3). Moreover, maximum acceleration was higher for males than for females (Table 3, Fig. A3) and was reduced in the second compared to the first generation (Table 3). A fluoxetine-induced increase of maximum acceleration emerged, but only when fish were not simultaneously exposed to high levels of 3,4-DCA (Table 3, Fig. 3B).

3.3. Anxiety-related and feeding behaviour

Cumulative time spent in the centre as measure for fish anxiety-related behaviour decreased upon fluoxetine-exposure, although this effect only emerged in the second generation of exposure to the compound (Table 4). Post-hoc analysis revealed that a difference in cumulative duration in the centre between fluoxetine-exposed fish and control fish (second generation) only emerged when fish were exposed to low levels of 3,4-DCA (Fig. 3C, Tukey $P = \mathbf{0.003}$). Likewise, mean distance to the centre was higher upon fluoxetine-exposure. However, this effect only emerged in fish of the first generation that were not exposed to the pesticide (Table 4, Fig. 3D, Tukey $P = \mathbf{0.003}$) and in second-generation fish that were exposed to low levels of 3,4-DCA (Table 4, Fig. 2C, $P = \mathbf{0.003}$). Moreover, fish exposed to low levels of 3,4-DCA on average were closer to the centre compared to control fish (Table 4, Fig. A4, Tukey $P = \mathbf{0.045}$).

Latency time to feed increased upon fluoxetine-exposure but only when fish were not simultaneously exposed to 3,4-DCA or when fish were exposed to the high levels of 3,4-DCA (Table 4, Fig. 4). No such effect emerged when fish were exposed to low levels of 3,4-DCA (Fig. 4, Tukey $P = 0.908$). Latency time to feed decreased in female fish of the second generation upon exposure to low levels of 3,4-DCA compared to fish that were not exposed to 3,4-DCA (Tukey $P = \mathbf{0.001}$) or to high levels of 3,4-DCA (Tukey $P = \mathbf{0.019}$)(Table 4, Fig. A5).

3.4. Repeatability of behavioural traits

All behavioural traits were significantly repeatable (Table 5).

4. Discussion

We studied the impact of full life-cycle exposure to a mixture of a conventional (3,4-DCA) and an emerging (fluoxetine) pollutant on life-history and behaviour across two generations in the killifish *N. furzeri*. The impact of fluoxetine exposure in combination with exposure to other chemical compounds has rarely been studied and this was the first attempt to study such effects across successive generations in a vertebrate model. Although the low sample size in the second generation - in particular for fish exposed to both pollutants simultaneously - limits a full interpretation of interactive effects across generations, our results show that both life-history and behaviour were affected by exposure to the pollutants. These effects differed between generations: fish of the second generation were smaller than first-generation fish when exposed to fluoxetine, and both fluoxetine and 3,4-DCA inhibited fecundity in the first but not second generation. In addition, pollutant effects were concentration-dependent and depended on each other: maximum swimming acceleration was increased by fluoxetine but not when fish were simultaneously exposed to 100 µg/L 3,4-DCA, and fluoxetine only increased latency time to feed when fish were not simultaneously exposed to 50 µg/L 3,4-DCA. Together, these findings illustrate the relevance of a multi-stressor approach across successive generations. While full life-cycle and multigenerational tests are impractical and costly for fish, this study delivers an effective demonstration that such studies are possible within a timespan of less than 6 months with the killifish *N. furzeri* as a model organism.

4.1. Life-history effects of fluoxetine and 3,4-DCA exposure

Consistent with our hypothesis, fluoxetine-exposed fish were smaller at 51 dph compared to control fish. Likely, a lower growth rate is due to a fluoxetine-induced reduction in food intake, as fluoxetine is known to have anorexigenic properties (McDonald, 2017). For instance, fluoxetine-supressed appetite has been shown in zebrafish (Shimada et al., 2012) and fluoxetine reduced the ability to capture prey in hybrid striped bass (*Morone saxatilis* × *M. chrysops*) (Gaworecki and Klaine., 2008). Similarly, a three-week exposure to environmentally relevant levels of fluoxetine reduced feeding in *N. furzeri* (Thoré et al., 2018a). The difference in total body length between fluoxetine-exposed fish and control fish did, however, not persist later in life (88 dph). Possibly, developmental constraints prevented control fish to grow further, allowing fluoxetine-exposed fish to catch up. Alternatively, and even though this change was the same for all fish, we cannot rule out that the change in feeding regime at 51 dph may have affected growth of control vs. fluoxetine-exposed fish differently. As another alternative explanation, fish may have habituated to fluoxetine-exposure. Habituation can entail compensatory responses in the brain that revert extracellular serotonin levels to the premedication equilibrium after long-term exposure to restore serotonin homeostasis (Andrews et al., 2015; Martin et al., 2019). Therefore, it would be interesting to monitor the effect of fluoxetine-exposure at multiple time points throughout an organisms' life. This is relevant not only because effects may be reversible after

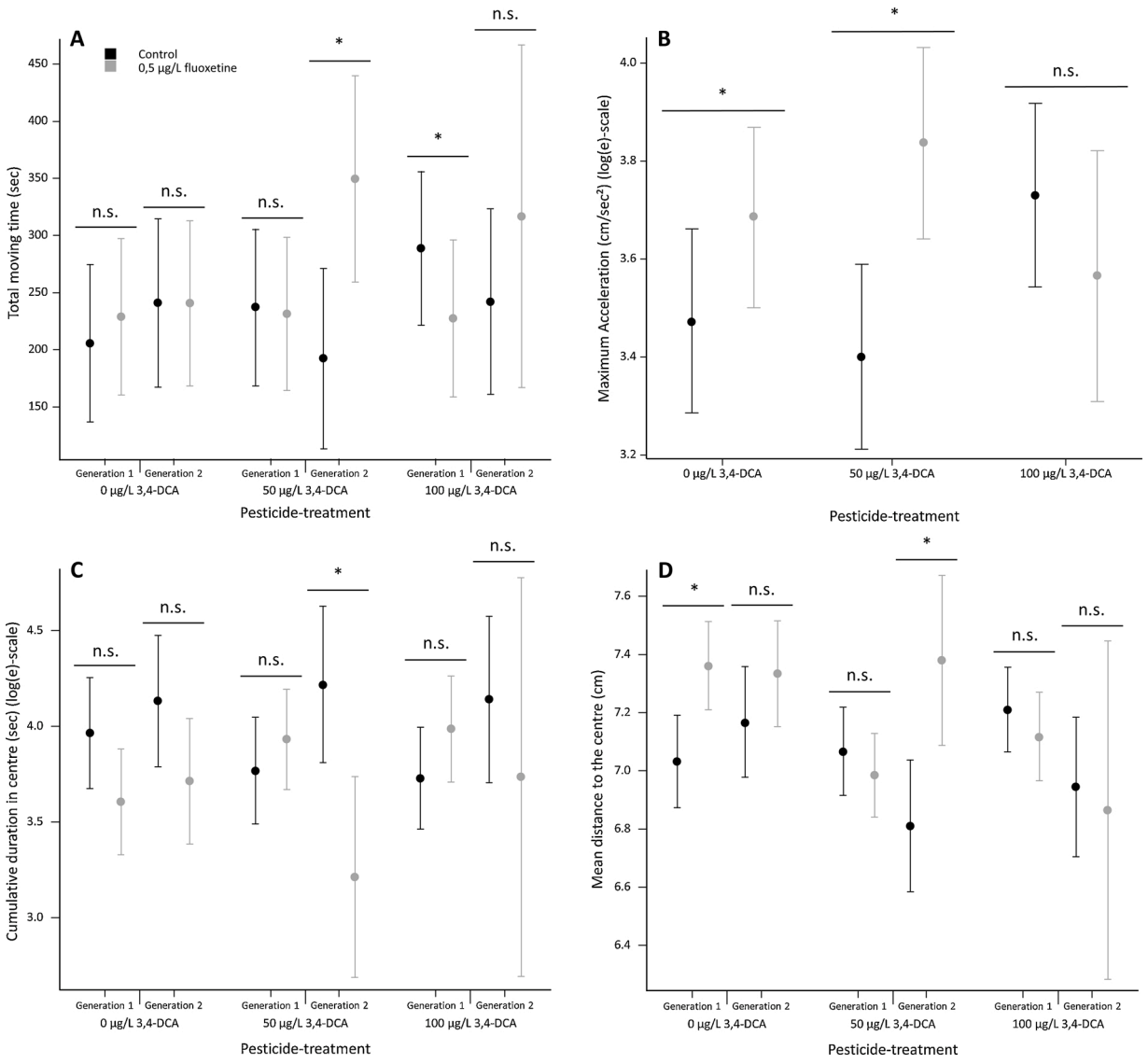

Fig. 3. Impact of pollutant-exposure on open-field activity (predicted effects). (A) The effect of fluoxetine- and pesticide-exposure on total time spent moving in the open-field test for fish of the first and second generation. (B) The effect of fluoxetine- and pesticide-exposure on maximum acceleration in the open-field test. (C) Effect of fluoxetine- and pesticide-exposure on total time spent in the centre and on (D) mean distance from the centre of the open-field arena as proxy for anxiety-related behaviour, for both generations. Significant differences are based on Tukey-corrected post-hoc tests and are indicated with an asterisk (*), non-significant differences are indicated with n.s. Whiskers delineate the upper and lower 95 % confidence limit.

long-term exposure, but also because there might be a lag phase between fluoxetine administration and visible effects of exposure (i.e., the therapeutic delay) (Andrews et al., 2015).

In contrast to our hypothesis, exposure to 3,4-DCA did not impact fish total body length. Possibly, the sensitivity range of *N. furzeri* exceeds the concentration range applied in the current study, at least for somatic growth. Alternatively and non-mutually exclusive, insufficiently long exposure could underlie the absence of a growth-response. In support, a recent study on *N. furzeri* found no impact of 3,4-DCA exposure at 100 µg/L after one and 7 weeks of exposure, whereas total body length was adversely impacted after 15 weeks of exposure (Philippe et al., 2019). Total body length is an important predictor of fitness with known effects on reproductive success (Polačik and Reichard., 2009), habitat selection (Yeager and Hovel., 2017) and susceptibility to predation (Nilsson and Brönmark, 2000). Therefore, the exact fitness consequences and ecological implications of fluoxetine- and 3,4-DCA-exposure in the wild remain unclear and should be subject to future research.

While fluoxetine exposure was associated with decreased fecundity in the current study (realised concentration of 0.152 µg/L), its effects on sexual reproduction in fish appear to be inconsistent. Lister et al. (2009), for instance, showed that egg production in zebrafish was inhibited under environmentally relevant levels of fluoxetine for 7 days. Other studies, however, reported no effect or even increased reproduction due to fluoxetine. For instance, egg production of Japanese medaka (*Oryzias latipes*) was not affected after four weeks of fluoxetine exposure (Foran et al., 2004) and 14-weeks exposure at higher concentrations of 0.5 and 5 µg/L even doubled reproductive output in *N. furzeri* in a previous study (Thoré et al., 2020). Differences between studies are likely due to different exposure regimes, species-specific sensitivity to fluoxetine and methodological differences (Martin et al., 2019). That being said, variation between studies hampers reaching robust conclusions on the environmental impact of fluoxetine, and developing standardised and relevant testing is therefore necessary (Sumpter et al., 2014; Thoré et al., 2018a).

Table 4
Model output of variables related to fish anxiety-related and feeding behaviour.

Effect	Cumulative duration in the centre		Mean distance to the centre		Latency time to feed	
	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value
Sex	−0.068	1.000	−0.035	1.000	0.106	0.765
Fluoxetine	6.052	**0.017**	3.453	0.072	12.147	**0.002**
Pesticide	0.360	0.845	8.099	**0.029**	6.591	0.056
Generation	−0.062	1.000	0.160	0.712	3.383	0.084
Cohort	5.992	**0.015**	4.235	0.051	10.479	**0.004**
Total length	0.495	0.509	−0.070	1.000	2.097	0.157
Sex:Fluoxetine	0.378	0.570	1.847	0.179	0.422	0.543
Sex:Pesticide	1.698	0.437	0.290	0.890	1.484	0.516
Fluoxetine: Pesticide	1.069	0.621	3.388	0.210	7.808	**0.035**
Sex:Generation	0.482	0.512	0.828	0.386	0.252	0.647
Fluoxetine: Generation	6.951	**0.011**	1.379	0.262	−0.062	1.000
Pesticide: Generation	1.327	0.566	2.831	0.265	7.223	**0.048**
Sex:Fluoxetine: Pesticide	0.603	0.748	0.336	0.861	0.461	0.838
Sex:Fluoxetine: Generation	0.180	0.694	0.197	0.666	0.736	0.417
Sex:Pesticide: Generation	1.056	0.622	1.844	0.446	15.450	**0.001**
Fluoxetine: Pesticide: Generation	5.619	0.080	9.942	**0.012**	3.091	0.251
Sex:Fluoxetine: Pesticide: Generation	2.109	0.340	1.071	0.632	0.914	0.688

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

As expected, exposure to 3,4-DCA reduced fecundity. Previously, Philippe et al. (2019) could not demonstrate any impact of lifelong 3, 4-DCA exposure on fecundity in *N. furzeri* at the same concentrations as the ones applied in the current study. However, while Philippe et al. (2019) exposed fish across their entire life, in the current study exposure started at 51 dph only. Potentially, exposed sexually non-active individuals habituate to 3,4-DCA and because of that their reproduction is less impacted when fully developed.

4.2. Interactive pollutant effects on fish behaviour

The adopted experimental design allowed to examine potential interactive effects between fluoxetine and 3,4-DCA. However, several of these effects should be interpreted with care, given that 1) the lower sample size in the second generation may not provide adequate statistical power, 2) spurious effects may be expected due to the high number of endpoints and comparisons, and 3) several traits may covary and hence not be independent. Because this limits a full interpretation of potential interactive effects, not all results are discussed here at length.

That being said, our findings suggest that a number of pollutant-induced effects were dependent on each other, in a concentration-dependent way. For instance, fluoxetine increased maximum swimming acceleration, but not when fish were simultaneously exposed to the highest concentration (100 µg/L) of 3,4-DCA. The finding that fluoxetine stimulates rather than inhibits swimming acceleration may not be unexpected, given that fluoxetine has anxiolytic properties that may stimulate fish swimming behaviour (Martin et al., 2017) and given that fluoxetine leads to higher levels of extracellular serotonin, which is important for the generation rather than inhibition of fish locomotion (McDonald, 2017). It is interesting, however, that these effects only emerged in single exposure treatment (24.0 % increase compared to

Table 5
Repeatability values for the measured behavioural traits. (CI = confidence interval).

Behavioural trait	Repeatability (R)	*P*-value	95 % CI
Distance travelled	0.296	<0.001	[0.209, 0.391]
Total time moving	0.312	<0.001	[0.219, 0.409]
Mean velocity	0.262	<0.001	[0.189, 0.351]
Maximum acceleration	0.088	<0.001	[0.039, 0.157]
Cumulative duration in centre	0.158	<0.001	[0.109, 0.234]
Mean distance to centre	0.169	<0.001	[0.118, 0.238]
Latency time to feed	0.316	<0.001	[0.248, 0.4]

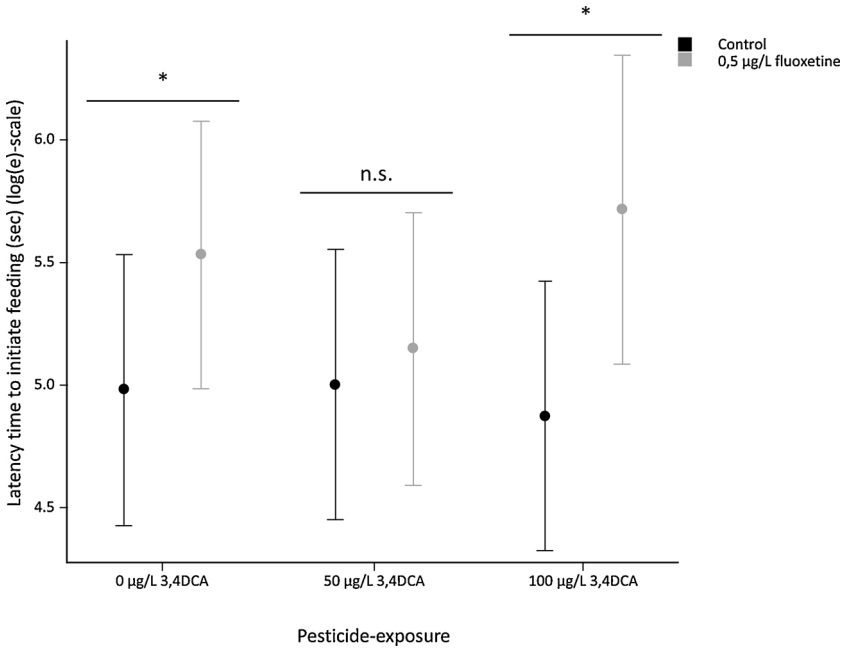

Fig. 4. Impact of pollutant-exposure on feeding behaviour (predicted effects). Effect of fluoxetine- and pesticide exposure on latency time to feed. Whiskers delineate the upper and lower 95 % confidence limit. Significant differences are based on Tukey-corrected post-hoc tests and are indicated with letters or an asterisk (*), non-significant differences are indicated with n.s.

control) or upon simultaneous exposure to a low concentration (50 µg/L) of 3,4-DCA (54.7 % increase compared to control), and that the effect seems more pronounced in the latter case. Although speculative, this observation could reflect a hormetic effect as a widely observed (yet debated (Kaiser, 2003)) phenomenon in ecotoxicology and could be interpreted as a coping mechanism to counteract low levels of environmental stress and promote survival (Calabrese and Mattson., 2017). Alternatively, rather than signalling improved locomotor behaviour, higher maximum swimming acceleration may also reflect erratic swimming bouts, which is a common indicator of anxiety in fish (McDonald, 2017; Nowicki et al., 2014).

Furthermore, fluoxetine also increased latency time to feed (average increase of 101.7 %), but only when fish were not simultaneously exposed to 50 µg/L 3,4-DCA. While the inhibitory effect of fluoxetine on fish feeding behaviour was not unexpected (see above), it is surprising that this effect did not emerge when fish were simultaneously exposed to a low concentration of 3,4-DCA. Potentially, a low level of 3,4-DCA-induced stress may have encouraged fish to feed (cf. compensatory effect), hence counteracting the inhibitory effect of fluoxetine.

Together, these findings illustrate the relevance of a multi-stressor approach given that the impact of exposure to one compound is dependent on additional stressors. Such interactions are not unusual in natural systems, and there is increasing evidence that organisms are affected through various interactive effects between diverse stressors (Galic et al., 2018). For instance, joint exposure of zebrafish to the antidepressant venlafaxine and increased temperature (+5 °C) increased standard metabolic rates whereas exposure to the stressors separately did not affect the metabolism (Mehdi et al., 2019). In contrast, fluoxetine increased activity levels in mosquitofish irrespectively of predator-induced stress (Martin et al., 2017).

Predicting the impact of multiple stressors is challenging as they may interact in complex ways (Galic et al., 2018). Still, understanding how a multiple-stressor environment affects the physiology of organisms is crucial to more accurately predict the environmental effects of pollution. From this perspective, it is worth noting that testing all possible stressor combinations that may occur in the environment is unfeasible, and should therefore not be aspired. However, we should aspire to increase our insight in the nature of interactive effects, i.e. when to expect them, how they are expressed, and how they are established. Ecotoxicological tests with a limited number of stressors may never reach full ecological relevance, but are nevertheless key to advance our understanding of multiple-stressor environments.

4.3. Changing sensitivity to fluoxetine and 3,4-DCA across generations

Several pollutant-induced effects differed between generations. For instance, fish of the second but not first generation were smaller (at 51 dph, 6.4 % smaller) when exposed to fluoxetine, suggesting that fluoxetine reduced juvenile growth only in the second generation of exposure. This may imply an increased sensitivity of fish across generations and is in line with findings on zebrafish (Vera-Chang et al., 2019). Further supporting this, brief ancestral exposure of zebrafish to fluoxetine altered exploratory and locomotor activity and reduced stress-induced cortisol levels for two and three successive unexposed generations, respectively (Vera-Chang et al., 2018).

Still, the opposite was found for fecundity: both fluoxetine and 3,4-DCA inhibited fecundity in the first (fluoxetine: 18.8 %, pesticide low: 23.3 %, pesticide high: 21.9 % reduction) but not in the second generation. Potentially, offspring habituation could contribute to these results. Since offspring were already exposed as gametes to the compounds, they may have developed an increased resistance. Alternatively, and although speculative, the observed responses may reflect a coping mechanism in which second-generation fish counteract the negative impact on reproduction by re-allocating energy from somatic growth to reproduction (hence, at the cost of a decrease in somatic growth). Thereby, fish might be able to rapidly respond to contaminant-exposure through shifts in life-history across generations.

4.4. Conclusions and future perspectives

Overall, the current study shows that pollutant effects may differ between generations and upon simultaneous exposure to several pollutants. Moreover, effects of long-term exposure may be reversible within the lifetime of an organism and not only impact traditional life-history but also behavioural traits. Such effects are, however, not detected using current standard ecotoxicological tests that generally focus on deleterious effects of acute exposure to single compounds. Although these challenges for ecotoxicology are increasingly recognised, so far no standardised ecotoxicological tests exist that effectively accommodate these problems while maintaining time- and cost-efficiency. The recent introduction of *N. furzeri* as a model organism for long-term ecotoxicological testing offers interesting opportunities to update current approaches.

In this study, we successfully used *N. furzeri* in a multi-stressor exposure experiment across two successive generations, focussing on both traditional life-history endpoints and more sensitive behavioural endpoints. The lower sample size for offspring of stressed fish in this study may point to an inherent challenge of the experimental design and should be taken into account in relation to statistical power when using such designs. In addition, ideally, when results are borderline and statistical power may be an issue, results should be shown to be repeatable to allow for a full interpretation (Harris et al., 2014). Nevertheless, the adopted multigenerational approach stands as a proof-of-principle that *N. furzeri* may be a useful model for such studies, especially when compared to classic fish models. We show that multigenerational exposure tests are possible within a timespan of less than 6 months with *N. furzeri*. In addition, eggs of this species can easily be stored for several years and simultaneously hatched to allow for age-synchronised testing, which further facilitates a multigenerational design. This also offers the possibility of including parental identity of fish, which would not only allow a stronger statistical design but also open interesting avenues for additional research questions.

Even though the current study showcases the advantages of *N. furzeri* for long-term ecotoxicological testing, a formal test-framework is needed before it can be used in official environmental risk assessment procedures. As a first step, existing OECD guidelines should be adapted to the use of *N. furzeri*. Examples include short-term reproduction assays (OECD test guideline 229), sexual development tests (OECD test guideline 234) and extended one-generation reproduction tests (OECD test guideline 240). Such tests may help to increase our understanding of interactive and multigenerational effects, which opens routes to more accurately translate results from laboratory-tests to the field.

Funding

This work was supported by Fonds Wetenschappelijk Onderzoek–Vlaanderen to E.S.J. Thoré (1S30518N) and T. Pinceel (12F0716N) and by the Excellence Center Eco- and Socio-Evolutionary Dynamics (grant PF/10/007 to C. Philippe).

CRediT authorship contribution statement

Eli S.J. Thoré: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Floor Van Hooreweghe:** Methodology, Writing - original draft. **Charlotte Philippe:** Writing - original draft. **Luc Brendonck:** Supervision. **Tom Pinceel:** Writing - original draft.

Declaration of Competing Interest

The authors declare no declaration of interest.

Acknowledgments

We are grateful to M. Reichard and coworkers for providing the parental fish for this study. We furthermore thank the reviewers for their constructive comments on the manuscript. The authors declare no competing financial interest.

Appendix A

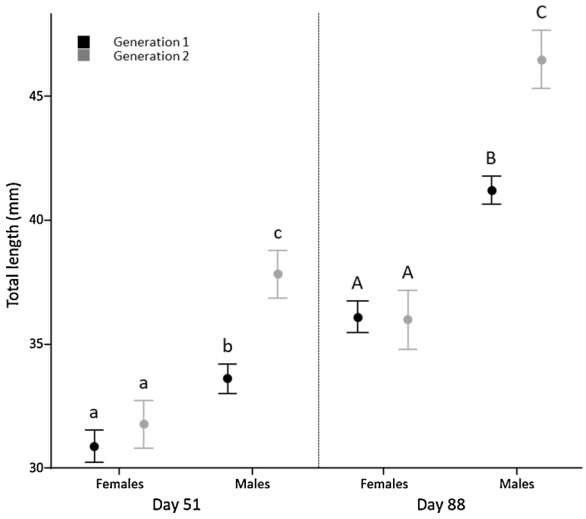

Fig. A1. Total body length at 51 dph (left panel) and 88 dph (right panel) for males and females per generation. Whiskers delineate the upper and lower 95 % confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests.

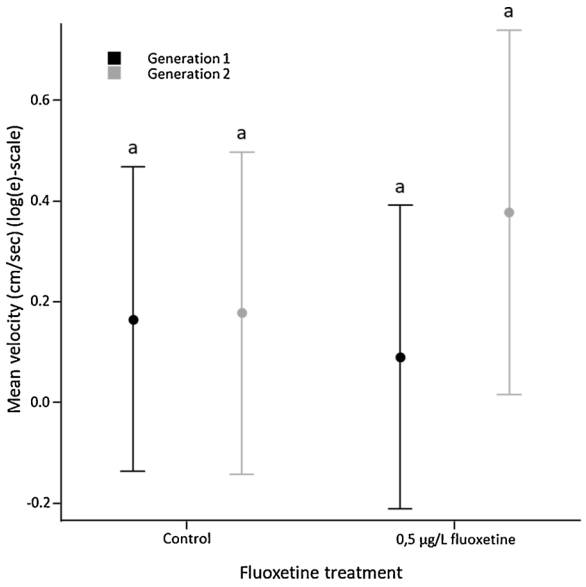

Fig. A2. Impact of fluoxetine on mean velocity in the open-field test for both generations. Whiskers delineate the upper and lower 95 % confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests.

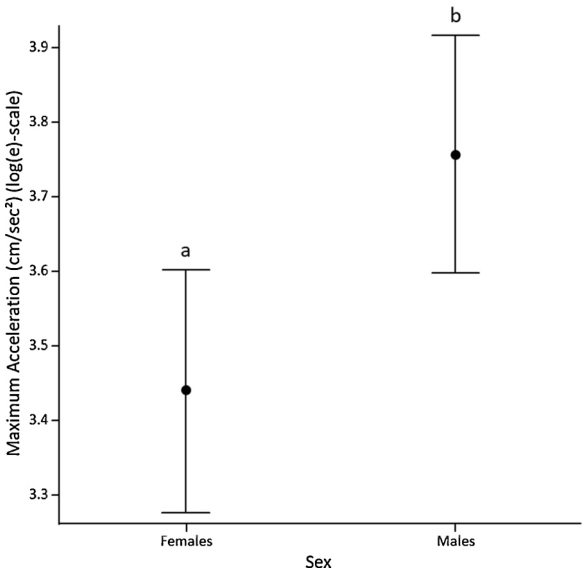

Fig. A3. Maximal acceleration (cm/sec^2) in the open-field test for females and males. Whiskers delineate the upper and lower 95 % confidence limit. Significant differences are based on Tukey-corrected post-hoc tests and are indicated with letters.

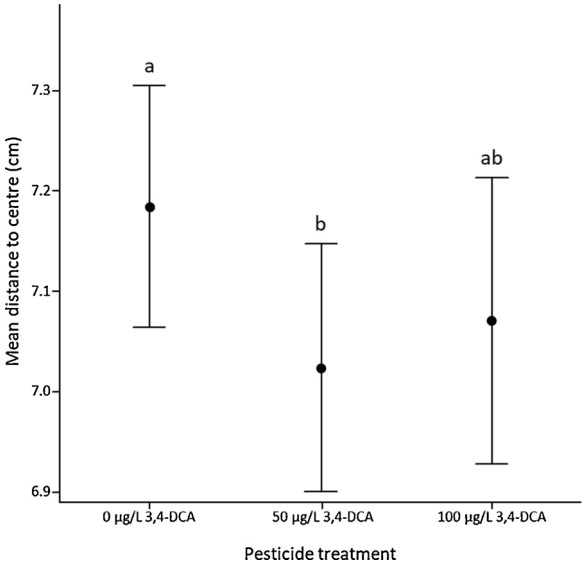

Fig. A4. Impact of pesticide exposure on mean distance to the centre in the open-field test. Whiskers delineate the upper and lower 95 % confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests.

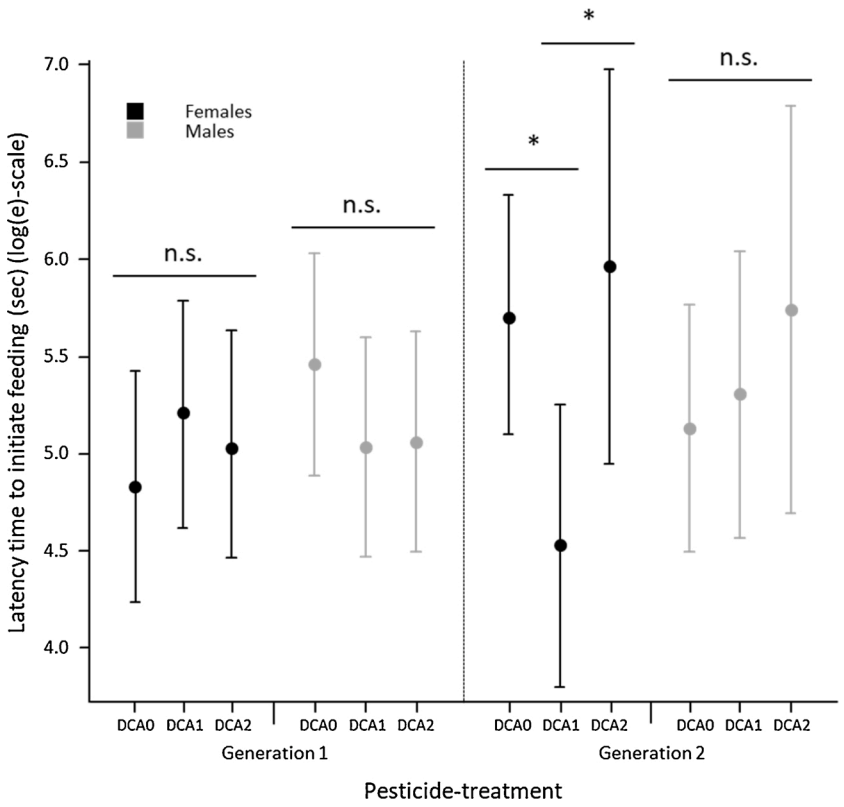

Fig. A5. Impact of pesticide-exposure on latency time to feed per sex per generation. Whiskers delineate the upper and lower 95 % confidence limit. Significant differences are based on Tukey-corrected post-hoc tests and are indicated with an asterisk (*), non-significant differences are indicated with n.s. (DCA0: 0 µ/L 3,4-DCA; DCA1: 50 µg/L 3,4-DCA; DCA2: 100 µg/L 3,4-DCA).

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