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DMSO effects larval zebrafish (*Danio rerio*) behavior, with additive and interaction effects when combined with positive controls

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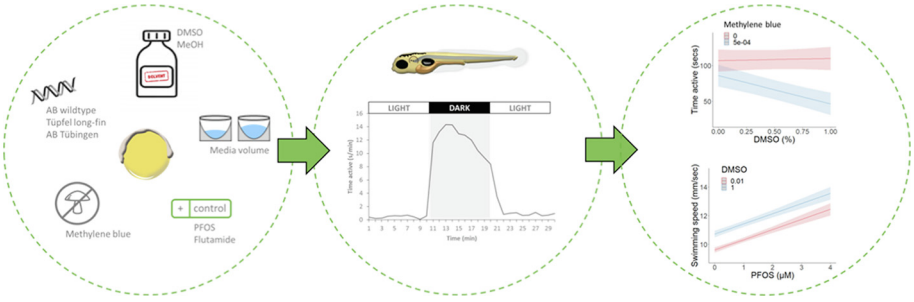
HIGHLIGHTS

- Solvents are frequently used during zebrafish toxicity testing but their effects are unknown.
- DMSO affected behavior at a concentration of ≥0.55%
- Different zebrafish strains showed different basal activity, but the same behavioral response to DMSO.
- DMSO had an additive and interaction effects on behavior when co-exposed with positive controls.

GRAPHICAL ABSTRACT



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ABSTRACT

Embryonic and larval zebrafish (*Danio rerio*) behavior is commonly used to identify neurotoxic compounds. Here, we investigated whether sub-lethal exposures to the common solvents dimethyl sulfoxide (DMSO, 0.01–1%) and methanol (MeOH, 0.01–1%), or the anti-fungal agent methylene blue (MB, 0.0001–0.0005%), can influence larval behavior in a simple light/dark paradigm conducted in 96-well plates. In addition, we tested whether the media volume within the behavioral arena or the zebrafish strain, AB wild type, AB Tübingen (AB/TU), or Tüpfel long-fin (TL), could also influence larval behavior. Following the single exposures, we co-exposed larvae to DMSO and either MB or two other compounds with known behavioral effects in larval zebrafish, flutamide and perfluorooctanesulfonic acid (PFOS). We found ≥0.55% DMSO and 0.0005% MB significantly affected larval behavior, but there was no effect of MeOH. Similarly, TL showed less movement compared to AB and AB/TU strains, whereas lower media volumes also significantly reduced larval movement. However, all strains responded similarly to DMSO and MB. In the co-exposure studies, we found either additive or interaction effects between DMSO and either MB, flutamide, or PFOS, depending on the behavioral endpoint measured. In addition, media volume had no effect on the DMSO concentration response curve, but again we observed additive effects on behavior. In conclusion, methodology can lead to alterations in baseline locomotor activity and compounds can have additive or interaction effects on behavioral endpoints. However, we found no evidence that strain effects should be a concern when deciding on solvents for a simple light/dark behavioral test in larval zebrafish.

1. Introduction

Since the establishment of the fish acute toxicity test (AFT) and the fish embryo acute toxicity test (FET) (OECD, 1992, OECD, 2013), multiple studies have been conducted using the zebrafish as a

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model organism. These studies have not only helped to determine the maximum tolerable concentrations of a broad spectrum of chemicals and other agents, but have also established guidelines for the testing of these substances (Belanger et al. 2013, Dang et al. 2017, Lammer et al. 2009, Scholz et al., 2014). In more recent years, behavioral endpoints are increasingly used as sub-lethal alternative endpoints to the traditional fish embryo toxicity test in ecotoxicology and chemical screening (Hellou 2011, Legradi et al. 2015). Of particular interest is the translational aspect of these studies, as zebrafish share a similar genome, brain structure, and neurochemical system as mammals (Best et al. 2008, Gerlai 2010, Kokel and Peterson 2008, Levin et al. 2007). Furthermore, zebrafish larvae show behavioral profiles similar to mammalian models following exposure to neuroactive drugs (Irons et al. 2010) making zebrafish ideal for comparative studies of neurotoxic effects (Legradi et al. 2018). The stereotypical behavior of zebrafish is well described, and many behavioral tests have been developed to evaluate the effects on sensory, motor, and cognitive behavior (Egan et al. 2009, Gerlai 2003, Miklosi and Andrew 2006). Behavioral tests that have been employed in zebrafish larvae include the thigmotaxis test (preference for the outer limits of a defined arena, Schnörr et al., 2012), the escape or avoidance test (Pelkowski et al., 2011), the acoustic test (Burgess and Granato 2007), the locomotor assay (Giacomini et al. 2006, Legradi et al. 2015), and the light/dark transition test. The light/dark transition assay is characterized by alternating dark and light periods and it has been established that during the dark periods zebrafish larvae move more than during the light periods (MacPhail et al. 2009).

Due to the increased use of zebrafish embryos and larvae in behavioral assays in toxicology and pharmacology, more attention is been given to the effect of solvents that are used during experimental procedures. Zebrafish embryos and larvae are primarily exposed to an aqueous solution containing the test compounds. However, the low water solubility of many compounds requires the use of solvents to accelerate the dilution process (Hutchinson et al. 2006). The most commonly used solvents in toxicology for the administration of chemicals are dimethyl sulfoxide (DMSO), ethanol, and methanol (MeOH). Studies have shown that different concentrations of the solvents can have an effect on their own on the behavior of zebrafish. For example, high concentrations of ethanol can cause hypoactivity whereas lower concentrations cause hyperactivity in larval zebrafish (de Esch et al. 2012, Lockwood et al. 2004) and DMSO at concentrations between 0.01 and 0.1% can have observable locomotor effects (Chen et al. 2011). The Organization of Economic Co-operation and Development (OECD) has specified a maximum solvent concentration of 0.1 ml/L (0.01% v/v) for aquatic tests (OECD, 1992, OECD, 2013), but standardization of this concentration in toxicity tests is still lacking.

Further to the use of solvents in toxicity testing, another common aspect of using zebrafish is the control of fungal pathogens. Here, laboratories may use the antifungal agent methylene blue (MB) to clean fertilized embryos and/or prevent fungal outbreaks during larval production. Common laboratory techniques for zebrafish rearing suggest that embryo and larvae are kept in embryo medium with MB at a concentration of 0.0002% for the first four days (Westerfield, 2007). To date, there is no information on the effects of MB on the behavioral response in larval zebrafish.

In addition to the solvent and/or use of antifungal agents, other aspects of larval zebrafish behavioral testing are not standardized. For example, a number of different strains are reported within the toxicology literature and these can exhibit different behavioral responses either at baseline levels or when exposed to different chemicals. For instance, larvae belonging to the AB strain were more active during a light/dark assay when compared to larvae of the Tüpfel long fin (TL) strain at days 5 and 6 post fertilization whereas this activity was reversed at day 7 (de Esch et al. 2012). Regarding strain effects on toxic responses, Pannia et al. (2014) observed that zebrafish adults belonging to the TU strain appeared to be more tolerant to ethanol treatment since only the WIK strain showed a dose- and time- dependent decrease in swimming duration following exposure. Furthermore, the physiology of zebrafish strains differ, as AB and TL larvae have differences in hypothalamus-pituitary-interrenal axis activity, expression of neurodevelopment and immune system related genes, and baseline levels of cortisol (van den Bos et al., 2017). In addition, various aspects of the larval behavioral test are known to influence baseline behavior, such as age, size of well, light conditions (Emran et al. 2008, Padilla et al., 2011), rearing conditions (Zellner et al., 2011), and the time of the day the assay took place (MacPhail et al. 2009). Differences in some of these variables can also lead to differences in behavioral outcomes during toxicity testing. For example, 10 μM bisphenol A was found to induce either hyper- or hypo-activity in larval zebrafish depending on the arena size used during testing (Fraser et al. 2017a). One aspect that has yet to be investigated is the volume of the media within a given behavioral arena. For example, zebrafish are commonly tested in 96 well plates, but the media volume can vary between 100 (Noyes et al. 2015) to 500 μl (de Esch et al. 2012).

Following the need for standardization of experimental procedures, we carried out a behavioral assay with zebrafish larvae using two of the most common solvents, DMSO and MeOH, as well as the antifungal agent MB. These compounds were evaluated at sub-teratogenic concentrations for effects on behavior applying a commonly used larval behavior test with three strains of zebrafish. In addition, we also evaluated the use of different media volumes for effects on behavior. Following this, we investigated whether DMSO and media volume could have interaction effects on behavior, as well as co-exposures between DMSO and two positive controls for larval locomotion, flutamide and perfluorooctanesulfonic acid (PFOS).

2. Materials and methods

2.1. Chemicals

DMSO (purity, >99.7%, CAS number 67-68-5), MeOH (purity, ≥99.9%, CAS number 67-56-1), MB (dye content, ≥82%, CAS number 122965-43-9), flutamide (purity, ≥99%, CAS number 13311-84-7) and PFOS (purity, ≥98%, CAS number 2795-39-3), were purchased from Sigma-Aldrich. Stock solutions of flutamide and PFOS were prepared in DMSO. Fresh stock solutions of flutamide were made on the day of testing whereas the PFOS stock solution was stored at − 20 °C.

2.2. Fish husbandry

The study was performed at The Norwegian University of Life Sciences (NMBU), Oslo, Norway, that is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway.

AB wild-type (AB), Tüpfel long fin (TL), and AB/Tübingen (AB/TU) zebrafish were maintained at 28 ± 1 °C under a 14:10 light/-dark photoperiod. Animal care was done in accordance with the local protocols. To generate embryos, adults were placed in spawning tanks in the afternoon and spawning occurred the following

morning when the lights turned on (08:00). The embryos were collected (09:00) and maintained in sterile embryo media (60 μg/ml Instant Ocean® sea salts) until the time of exposure.

2.3. Exposures

The exposure concentrations and the strains used to test certain compounds can be found in Table 1. Fertilized embryos were transferred into clear polystyrene 96-well plates (Nunc™ MicroWell™) and continuously exposed under static conditions from 6 hpf until the time of testing at 98–102 hpf (between 11:00–15:00). For DMSO and MeOH, five nominal concentrations ranging from 0.01 to 1% (1.41–141 mM for DMSO and 2.47–247 mM for MeOH) were tested. These concentrations are below the minimum effect concentrations for teratogenicity which are 2.0–2.5% for 24–168 hpf larvae (Maes et al. 2012). For MB, three nominal concentrations of 0.0001, 0.0002, and 0.0005% (3.1, 6.3, and 15.6 μM, respectively) were chosen based on general guidelines for zebrafish (Westerfield, 2007). For media volume, four volumes ranging between 50 and 200 μl were chosen based on volumes frequently used within the literature (Khezri et al. 2017, MacPhail et al. 2009, Noyes et al. 2015). For mixture experiments, we compared the concentration response to flutamide (FLU) between 1 and 10 μM and PFOS between 0 and 4 μM, the range in which we previously found FLU (Fraser et al. 2017b) and PFOS (Khezri et al. 2017) to increase swimming speeds, when co-exposed to 0.01 or 1% DMSO. We compared the concentration response of DMSO between 0.1 and 1% when using only 50 or 200 μl of media. Finally, we compared the concentration response to DMSO between 0.1 and 1% when in the presence or absence of 0.0005% MB. Each scenario described above was repeated three to four times using independent batches of larvae on different days. For DMSO, MeOH, and MB, each of the three strains of zebrafish were assessed separately. Prior to and following exposure, embryos were reared in an incubator at 28 ± 1 °C. The light cycle within the incubator was 14:10 light/dark (lights on 07:30/lights off 21:30). For the single exposures, all groups were spread equally on each row and column over one 96 plate/replicate. For the co-exposures, all groups were spread equally over each row and column in two 96 well plates/replicate.

2.4. Larval behavior

Behavioral tests were conducted using a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France). Behavioral screening was undertaken at 98–102 hpf that was between 11:00 and 15:00. Previously, we have found this age/time period to produce repeatable behavioral effects with many compounds (i.e. Fraser et al. 2017ab). Larval locomotion behavior, the cumulative distance travelled and the time spent active, were simultaneously measured for all larvae on a given well-plate during a light–dark cycle that lasted for a total of 30 min and consisted of 20 min of light and 10 min of darkness. This protocol has been used in our laboratory (Fraser et al. 2017a) and others (Fetter et al. 2015), but there is no standard protocol for the length or number of cycles (e.g. Noyes et al. 2015). The mean swimming speed was calculated by dividing the cumulated distance travelled by the total time spent active. The light level was set to 100% on the ViewPoint software (7.45 Klux, TES 1337 light meter). Infrared light (850 nm) tracks larval activity during the "dark" periods. The threshold for determining movement was set at 5 mm/sec. The larvae were inspected under a stereomicroscope immediately after behavioral testing in order to identify dead or deformed (coagulated, unhatched, spinal aberrations, yolk sac or cardiac edema, aberrations in pigmentation, swim bladder development, and/or loss of equilibrium) larvae. The maximum % of a batch discounted for behavioral analysis based on these criteria was 16% (Table 1). For FLU and PFOS, the number of larvae excluded from the behavior analysis due to these criteria ranged between 0 and 4% and 5–9% depending on concentration, respectively, confirming no signs of teratogenicity for either compound.

2.5. Statistical analysis

Behavioral data were transferred to R version 3.5.3 (R Development Core Team 2018, http://www.r-project.org). All dead and deformed larvae were discounted for behavioral analyses. For all test scenarios, only motility during the dark phase was analyzed as movement was minimal during the light periods. We used linear mixed effect (LME) models within the "nlme" package of R to assess behavior. The dependent variable was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed (calculated as the cumulated distance travelled/cumulated time spent active), with concentration set as a continuous variable, strain as a categorical independent variable, and replicate as a random effect. To compare whether strain influenced the behavioral response to DMSO, MeOH, or MB, we compared two models using the Bayesian Information Criteria (BIC) to identify the model with the lowest BIC score and considered this the "true" model (Aho et al. 2014). One model allowed for an interaction between strain and the tested compound (i.e. strain×DMSO), evidence of an interaction, the other allowed no interaction (i.e. strain+DMSO), evidence for no

Table 1
Overview of the experimental design. *Those individuals excluded from the statistical analysis were either dead or deformed according to the criteria detailed in the methods.

Dose response	Concentrations	Co-exposure scenario	Strains tested	Individuals/group (replicates)	Excluded individuals*/replicate (%)
–	–	–	AB, AB/TU, TL	16 (3)	AB, 6, 0, 0: AB/TU, 6, 12, 0: TL, 6, 0, 6
Dimethyl sulfoxide (DMSO)	0.00, 0.01, 0.10, 1.00%	–	AB, AB/TU, TL	16 (3)	AB, 0, 0, 9: AB/TU, 2, 9, 0: TL, 2, 3, 0
Methanol	0.00, 0.01, 0.10, 1.00%	–	AB, AB/TU, TL	16 (3)	AB, 0, 0, 3: AB/TU, 2, 8, 3: TL, 0, 0, 0
Methylene blue (MB)	0.0000, 0.0001, 0.0002, 0.0005%	–	AB, AB/TU, TL	16 (3)	AB, 2, 5, 0: AB/TU, 8, 12, 0: TL, 9, 6, 16
Media volume	50, 100, 150, 200 μl	–	AB	16 (4)	3, 2, 9, 2
Flutamide	0.0, 1.0, 3.2, 5.5, 7.8, 10.0 μM	0.1 or 1.0% DMSO	AB	12 (3)	1, 1, 3
DMSO	0.00, 0.10, 0.32, 0.55,0.78, 1.00%	0 or 0.0005% MB	AB	12 (3)	3, 1, 2
DMSO	0.00, 0.10, 0.32, 0.55,0.78, 1.00%	50 or 200 μl media volume	AB	12 (3)	2, 4, 2
Perfluorooctanesulfonic acid	0, 0.25, 0.50, 1.00, 2.00, 4.00 μM	0.1 or 1.0% DMSO	AB	12 (4)	6, 6, 8, 7

interaction. A null model that included the random effect was included as a third model. The model with the lowest BIC score was run. A final model used those larvae exposed to 0–1% DMSO in the co-exposure studies and 200 μl of media volume (i.e. pooled data from Fig. 1C–D), to determine the lowest effect concentration for DMSO. The "Anova" command within the "car" library was used to extract the results for the main effects whereas the "lsmeans" command within the "emmeans" library was used as a post-hoc test to compare groups against one another while adjusting for the means of other factors within the model (Lenth, 2016). Type II sum of squares were used for models without interactions, whereas main effects were calculated using type III sum of squares when interactions were present within the final model. The R^2 of the model was determined using the command "r.squaredGLMM" that returns the marginal and conditional R^2 that represent the variance explained by the fixed actors alone excluding the random effect and the variance of the entire model including the random effect, respectively (Nakagawa and Schielzeth 2013). The raw data (Behaviour.csv) can be found within the supplementary material as can plots of the raw data (Supplementary figures.pdf). For all models, examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. standardized residuals vs fitted values). Five models were corrected for heteroscedasticity using the command "weights = varPower()", DMSO and strain (distance

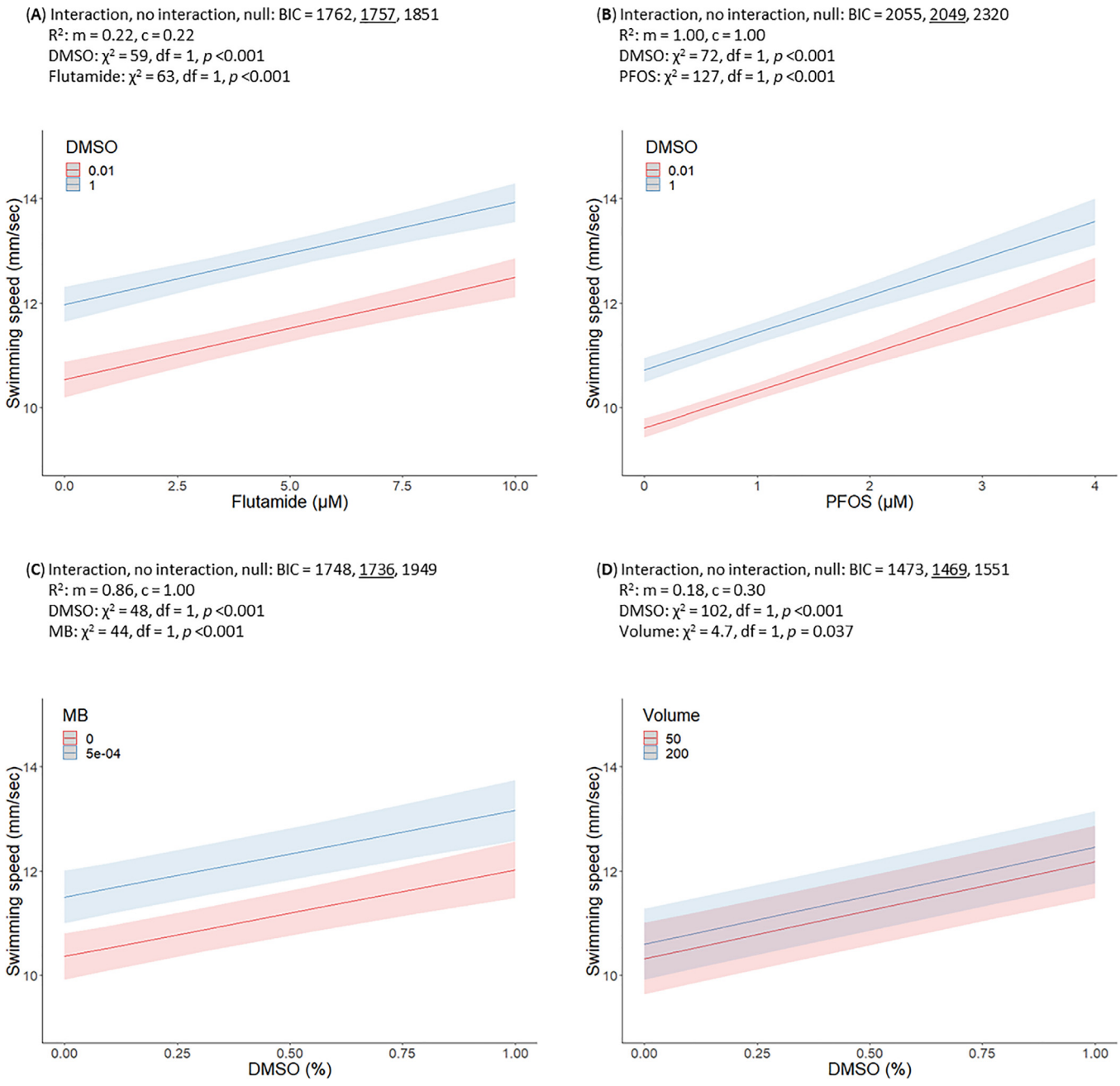

Fig. 1. Dose responses following co-exposure studies in larval zebrafish. (A) Dimethyl sulfoxide (DMSO) co-exposed with flutamide. (B) DMSO co-exposed with perfluorooctanesulfonic acid (PFOS). (C) DMSO co-exposed with methylene blue (MB). (D) DMSO and media volume. The model with the lowest BIC score (underlined) is presented along with the magrinal (m) and conditional (c) R^2. Results are those of linear mixed effect models and include regression lines ± 95% CI. N = 32–47 group^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

moved and time active), DMSO co-exposed with MB (time active and swimming speed), and PFOS co-exposed with DMSO (swimming speed). Significance was assigned at $p = < 0.05$.

3. Results

All results are summarized in Table 2

3.1. Strain and single exposures

Strain effects were apparent with TL showing significantly less locomotion compared to AB and AB/TU. However, there were no interactions between strain and any of DMSO, MeOH, or MB, on behavioral endpoints as all models without the interaction had a lower BIC score than those with the interaction.

There was a significant reduction in the time spent active, but an increase in swimming speed, in those larvae exposed to 1% DMSO compared to the control. When using the data from the co-exposure studies, the lowest observed effect concentration was 0.55% DMSO (lsmean post hoc, df = 412, $t = -4.3$, $p = 0.003$). MeOH had no effect on any behavioral endpoint. The distance moved and time active were significantly reduced by 0.0005% MB, but swimming speed was significantly increased, compared to lower concentrations. Greater media volume led to significant increases in the distance moved and time spent active, but there was no effect on swimming speed.

3.2. Interactions between toxins and methods

Although we saw the expected dose dependent increase in swimming speed with FLU, PFOS, DMSO, and MB, there were no interactions between flutamide and DMSO (Fig. 1A), PFOS and DMSO (Fig. 1B), DMSO and MB (Fig. 1C), or DMSO and media volume (Fig. 1D).

For the distance moved and the time active there was no interaction or addition between DMSO and either FLU (Distance moved: BIC scores, 6421 [interaction], 6418 [no interaction], 6429 [null], R^2 = 0.05 [marginal] and 0.08 [conditional], DMSO χ^2 = 23, df = 1, $p < 0.001$, FLU χ^2 = <1, df = 1, p = 0.967; Time active: BIC score, 4337 [interaction], 4334 [no interaction], 4333 [null]) or media volume (Distance moved: BIC score, 6190 [interaction], 6192 [no interaction], 6182 [null]; Time active: BIC scores, 4099 [interaction], 4102 [no interaction], 4094 [null]). In contrast, DMSO had an interaction effect with PFOS (Fig. 2AB) and MB (Fig. 2CD). Here, the distance moved and time active tended to be positively associated with PFOS when combined with 0.01% DMSO, but there as a negative association when combined with 1% DMSO. Similarly, the distance moved and time active tended to show a slight positive association with DMSO concentration in the absence of MB, but a negative association with DMSO concentration when in the presence of 0.0005% MB.

4. Discussion

We assessed various aspects of methodology relevant to toxicity testing on zebrafish locomotor activity using a high-throughput methodology. We found that DMSO, MB, media volume, and strain all had an effect on the behavioral response of larval zebrafish. In co-exposures between DMSO and MB, flutamide, or PFOS, these compounds either acted independently of one another, or interacted with one another, depending on the locomotor endpoint measured. Similarly, DMSO and media volume had additive effects. These results have important implications when trying to translate larval behavioral studies or comparing studies between laboratories, regarding toxicity testing.

Table 2
Behavioral data and statistical results for larval zebrafish of different strains following exposures to carrier solvents and/or media volume. The data are means with the upper and lower ranges based on lsmeans in parentheses, $N = 41–48$ group^{-1}. For model comparison, the BIC score is reported. The model with the lowest BIC score is underlined, and the statisitics are from lme models, unless the null model had the lowest BIC score, and include the marginal (m) and conditional (c) R^2 of the chosen model. Different subscript letters within rows indicate significant group effects (lsmean post hoc).

Parameter	Strain/Concentration/Volume				Model comparison (BIC score)	R^2 (m, c)	Model results (χ^2, df, p)		
Strain	AB	AB/TU	TL		Strain, null		Strain		
Distance moved (mm)	951 (468–1435)^a	853 (367–1339)^a	628 (143–1113)^b		2029, 2040	0.12, 0.30	23, 2, <0.001***		
Time active (seconds)	99 (52–145)^a	87 (40–133)^a	64 (17–110)^b		1381, 1397	0.14, 0.32	28, 2, <0.001***		
Swimming speed (mm/s)	9.54 (8.85–10.23)	9.71 (9.00–10.41)	9.86 (9.16–10.56)		389, 382	–	–		
Dimethylsulfoxide (DMSO)	0.00%	0.01%	0.10%	1.00%	Strain×DMSO, Strain+DMSO, null		Strain	DMSO	
Distance moved (mm)	845 (744–946)	892 (788–996)	908 (803–1014)	835 (734–935)	8241, 8229, 8212	–	–	–	
Time active (seconds)	81 (70–92)	83 (72–94)	84 (74–95)	69 (59–80)	5550, 5540, 5538	–	–	–	
Swimming speed (mm/s)	10.54 (9.81–11.27)^a	10.66 (9.93–11.39)^a	10.75 (10.02–11.48)^a	12.55 (11.82–13.28)^b	2165, 2154, 2274	0.20, 0.37	1, 2, 0.480	155, 1, <0.001***	
Methanol (MeOH)	0.00%	0.01%	0.10%	1.00%	Strain×MeOH, Strain+DMSO, null		Strain	MeOH	
Distance moved (mm)	1162 (1039–1285)	1064 (940–1188)	1077 (954–1200)	1062 (939–1185)	8433, 8421, 8411	–	–	–	
Time active (seconds)	111 (98–124)	102 (89–115)	103 (90–116)	102 (89–115)	5729, 5717, 5704	–	–	–	
Swimming speed (mm/s)	10.37 (9.69–11.05)	10.54 (9.86–11.22)	10.49 (9.82–11.17)	10.49 (9.81–11.17)	1970, 1965, 1949	–	–	–	
Methylene blue (MB)	0.0000%	0.0001%	0.0002%	0.0005%	Strain×MB, Strain+MB, null		Strain	MB	
Distance moved (mm)	903 (778–1029)	864 (738–989)	897 (771–1022)	739 (613–865)	7948, 7936, 7934	–	–	–	
Time active (seconds)	91 (79–103)^a	88 (76–100)^a	90 (78–101)^a	70 (58–82)^b	5430, 5417, 5427	0.06, 0.15	2, 2, 0.406	28, 1, 0.001***	
Swimming speed (mm/s)	9.75 (9.41–10.09)^a	9.74 (9.41–10.08)^a	10.00 (9.66–10.34)^a	10.53 (10.19–10.87)^b	1865, 1856, 1875	0.11, 0.14	13, 2, 0.001***	33, 3, 0.001***	
Media volume	50 µl	100 µl	150 µl	200 µl	Volume, null		Volume		
Distance moved (mm)	669 (415–920)^a	851 (601–1100)^b	952 (701–1202)^{bc}	1025 (774–1276)^c	3623, 3648	0.10, 0.20	34, 3, 0.001***		
Time active (seconds)	63 (44–82)^a	80 (61–98)^b	91 (72–110)^{bc}	100 (81–118)^c	2427, 2461	0.14, 0.20	44, 3, 0.001***		
Swimming speed (mm/s)	10.30 (9.20–11.41)	10.48 (9.39–11.57)	10.23 (9.13–11.32)	10.30 (9.20–11.41)	828, 823	–	–		

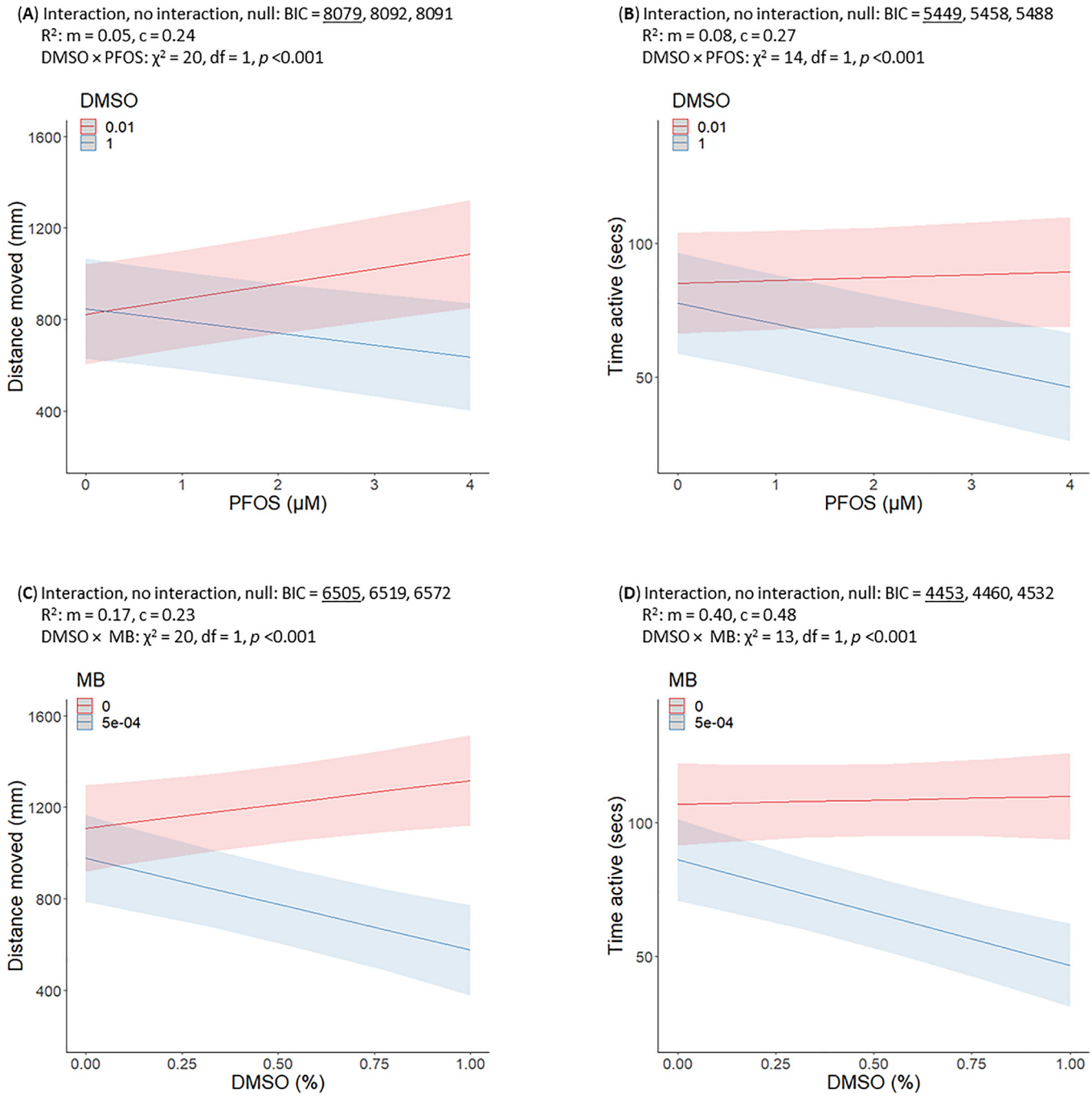

Fig. 2. Dose responses following co-exposure studies in larval zebrafish. The distance moved (A) and the time active (B) for dimethyl sulfoxide (DMSO) co-exposed with perfluorooctanesulfonic acid (PFOS). The distance moved (C) and the time acitve (D) for DMSO co-exposed with methylene blue (MB). The model with the lowest BIC score (underlined) is presented along with the magrinal (m) and conditional (c) R^2. Results are those of linear mixed effect models and include regression lines ±95% CI. N = 32–47 group^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We found DMSO, but not MeOH, had sub-lethal effects on larval behavior, irrespective of the strain tested. Our lowest observed effect concentration of DMSO was 0.55%. Previous studies have also demonstrated that exposure to ≥0.01% DMSO led to hyperactivity in 144 hpf larvae (de Esch et al. 2012), but also adult zebrafish exposed to 0.05% DMSO for 3–4 min (Sackerman et al., 2010). The differences observed in the lowest effect concentrations of DMSO might be due to the different behavioral assays or age of the fish. Previous studies have shown that behavioral outcomes vary depending on larval age (de Esch et al. 2012, Fraser et al. 2017a, Padilla et al., 2011), and we previously found larval behaviour was less sensitive to endocrine disrupting compounds compared to the literature on adult behaviour and/or molecular endpoints (Fraser et al. 2017b). At the molecular and protein level, it has also been shown that DMSO at concentrations as low as 0.01% can affect the expression of genes that are related to metabolic, developmental, and other biological processes (Turner et al., 2012), and the expression of heat shock proteins (Hallare et al. 2006). In contrast, we found no behavioral alterations in zebrafish larvae exposed to MeOH up to a concentration of 1%. This agrees with a previous study by (Lockwood et al. 2004) which showed exposure to methanol up to 7 dpf at a concentration of

1.5% had no significant effect on swimming speed. However, at the physiological level, larval zebrafish may be more sensitive since 0.05% methanol significantly reduced the expression of CYP1A and inhibited EROD activity (David et al. 2012). A comparison of the teratogenic effects of DMSO and MeOH showed that for both solvents larvae were quite tolerant, exhibiting malformations at concentrations between 2 and 2.5% (Maes et al. 2012). Based on our current results, when testing the behavioral effect of substances using our protocol, we recommend DMSO not be used >0.3% whereas MeOH can be used at concentrations as high as 1%. However, it is clear from the available literature that other endpoints and behavioral protocols maybe more sensitive to the concentrations of DMSO and MeOH recommended for use with our behavioral paradigm.

We found behavioral effects of MB, with a lowest effect concentration of 0.0005% (15.6 μM). It is generally recommended that for zebrafish larvae rearing the concentration of MB should be equal to 0.0002% (6.3 μM, Westerfield, 2007) for which we found no behavioral effects. In a study conducted by Hedge et al. (2017), zebrafish larvae treated for 6 dpf with MB at concentrations up to 10 μM were not affected in terms of their locomotor activity or any of the developmental aspects examined (death, hatching rate, swim bladder inflation, or deformities). Another study found MB led to a hermetic response on memory retention in adult zebrafish tested in a t-maze (Echevarria et al. 2016). Compared to controls, fish exposed to 0.5 μM MB performed significantly better, fish that received 5 μM did not exhibit any differences, whereas fish exposed to 10 μM performed worst (Echevarria et al. 2016). Based on the results of the above-mentioned studies and our observations, we recommend using concentrations of ≤0.0002% MB (6.3 μM) for raising larvae destined for behavioral testing. However, it is noted we continuously exposed larvae to MB from 6 hpf until testing (98–102 hpf) whereas others may only briefly wash larvae in MB immediately after fertilization. Therefore, future work should address toxicity thresholds for shorter exposure periods.

Having observed effects of DMSO and MB alone, we expanded our study to evaluate whether these compounds would interact with one another or other positive controls. Here, we found our results depended on the endpoint measured. When assessing swimming speed, we found no interactions between DMSO and MB, or between DMSO and two positive controls, PFOS or flutamide. Instead, we found these compounds had additive effects when used in combination. In contrast, the distance moved and time active showed interaction effects with PFOS and MB, but not FLU. For example, the distance moved was negatively associated with increased DMSO in the presence of MB, but showed a slight positive association with DMSO in the absence of MB. Therefore, further tests are required to understand whether other endpoints, such as molecular pathways, protein expression, neuroanatomy, may be influenced by solvents or MB during testing.

As expected, we found strain effects on behavioral profiles as the TLs exhibited a general decrease in activity in relation to both the AB and the AB/TU strains. Our results agree with previous studies showing that the AB strain at ages 5 and 6 dpf show higher activity than larvae belonging to the TL strain (de Esch et al. 2012), but are in contrast to another study that reported higher activity in the TL strain compared to AB (van den Bos et al., 2017). However, these studies cannot be directly compared due to differences in methodology. For example, van den Bos et al. (2017) raised the larvae together in petri dishes, which is reported to increase activity compared to those larvae raised in isolation (Zellner et al., 2011), the latter being the method we employed. In addition, van den Bos et al. (2017) used 24 well plates compared to our study that used 96 well plates, and larger arena have been reported to generally increase locomotor activity (Fraser et al. 2017a). In addition, the same strain of fish coming from different laboratories may differ in their levels of genetic variation that may also influence behavior (Coe et al. 2009). Nevertheless, van den Bos et al. (2017) also reported elevated gene expression of several markers related to neurodevelopment in AB larvae compared to TL larvae suggesting AB larvae may develop faster, which may translate to a stronger increase in activity in response to changes between light and dark conditions. The same authors also showed that AB larvae have a higher baseline level of cortisol that is commonly associated with behavioral differences (van den Bos et al., 2017). Therefore, physiological differences in strains exist that could influence behavior.

We observed no interaction between DMSO and different strains of zebrafish. This means that for larval behavioral studies, DMSO may have little influence on different zebrafish strains. However, the behavioral outcome of the AB and TU strains were found to be differentially affected when zebrafish larvae were exposed to the NMDA receptor antagonist MK-801 (Liu et al. 2014). Similarly, Loucks and Carvan (2004) found the response of three zebrafish strains (EK, AB, TU) to different concentrations of ethanol during the first 6 days of development varied in terms of survival, neurocranial and craniofacial skeletal development, and cell death (Loucks and Carvan 2004). Similarly, strain effects on reproductive endpoints have been recorded in zebrafish exposed to endocrine disruptors (Brown et al. 2011, Söffker et al., 2012). Therefore, although there is evidence strain effects exist, they appear to be endpoint sensitive.

In accordance with previous work on arena size, we found the amount of media within a given arena effected baseline behavior. In general, locomotor activity decreased with decreasing media volume. Similarly, Padilla et al. (2011) found zebrafish larvae kept in a 24 well plate moved more than the larvae kept in 48 and 96 well-plates, although the level of activity did not differ between larvae kept in 96 and 48 well plates. Based on their results the authors hypothesized that the activity of larvae is related more to the circumference of the arena rather than the area of the well (Padilla et al., 2011). In our study, we kept the circumference of the arena consistent by using 96 well plates throughout, but changed the area available for larvae to move in by altering media volumes. As activity increased with increasing media volume, it may be that larvae move more due to the increase in available space. Our results agree with a previous study that larvae kept in deep wells were more motile than larvae kept in shallow wells (Ingebretson and Masino 2013). This should be taken into account when comparing behavioral outcomes from different studies that use different testing volumes. However, we observed no interaction between media volume and DMSO concentration, suggesting that for behavioral studies at least, shallower wells may not influence toxicity results.

In conclusion, locomotor activity was shown to be influenced by various aspects of methodology, such as solvent, the use of MB, media volume, and strain. These results show that basal locomotor activity can be influenced by methodology making the standardization of experimental parameters in behavioral testing essential in order for direct comparisons between laboratories. We found both additive and interaction effects between methodologies depending on the behavioral endpoint measured in response to positive controls. Following the identification of sources of variability in this study, but also those preceding it, these parameters should be tested within different laboratories and behavioral tests in order to work towards the standardization of protocols.

CRediT authorship contribution statement

Maria Christou: Investigation, Writing - original draft, Visualization. **Arturas Kavaliauskis:** Investigation, Writing - review &

editing. **Erik Ropstad:** Resources, Funding acquisition, Writing - review & editing, Supervision, Project administration. **Thomas William Kenneth Fraser:** Conceptualization, Investigation, Formal analysis, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

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