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Toxicant induced behavioural aberrations in larval zebrafish are dependent on minor methodological alterations

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Highlights

- The toxicants BPA and TBBPA impact on larval zebrafish behaviour
- The behavioural responses are modified by methodological approaches
- 10µM BPA induced hyper or hypo-activity, or no response, depending on methodology

Abstract

Alterations in zebrafish motility are used to identify neurotoxic compounds, but few have reported how methodology may affect results. To investigate this, we exposed embryos to bisphenol A (BPA) or tetrabromobisphenol A (TBBPA) before assessing larval motility. Embryos were maintained on a day/night cycle (DN) or in constant darkness, were reared in 96 or 24 well plates (BPA only), and behavioural tests were carried out at 96, 100, or 118 (BPA only) hours post fertilisation (hpf). We found that the prior photo-regime, larval age, and/or arena size influence behavioural outcomes in response to toxicant exposure. For example, methodology determined whether 10 µM BPA induced hyperactivity, hypoactivity, or had no behavioural effect. Furthermore, the minimum effect concentration was not consistent between different methodologies. Finally, we observed a mechanism previously used to explain hyperactivity following BPA exposure does not appear to explain the hypoactivity observed following minor alterations in methodology. Therefore, we demonstrate how methodology can have notable implications on dose responses and behavioural outcomes in larval zebrafish motility following identical chemical exposures. As such, our results have significant consequences for human and environmental risk assessment.

Keywords: endocrine disruption; neurotoxicology; bisphenol A; tetrabromobisphenol A; brain; locomotion

1. Introduction

The zebrafish (*Danio rerio*) is an established vertebrate model in developmental biology and is becoming increasingly popular as a tool for identifying neurotoxic compounds. In particular, a growing number of studies use larval zebrafish motility during light/dark cycles to test new and existing compounds for neurotoxicity (i.e. Jarema et al., 2015; Noyes et al., 2015). Importantly, the results from such tests share a general agreement with those of other vertebrate models and cell lines (Ali et al., 2012; Irons et al., 2010; Rubinstein et al., 2006; Ton et al., 2006), thereby demonstrating the potential for zebrafish larvae in toxicity screening. However, compared to rodent models, zebrafish behavioural research is still in its infancy and there is little standardisation between studies on larval motility. This is a concern, as little is known about how alterations in methodology may influence toxicity testing.

The most common test when using larval zebrafish is to assess motility during alternating periods of light and dark at approximately 120 hours post fertilisation (hpf). Here, one expects control larvae to show freeze behaviour during periods of bright light, but a sharp increase in motility following the onset of darkness (Jarema et al., 2015). However, activity levels are known to be influenced by the timing of the experiment. For example, larvae are typically reported to become more active in the light with increasing age (i.e. Esch et al., 2012) and the level of activity during the dark period is also known to change throughout the day (MacPhail et al., 2009). The reasons behind these observations are unclear, but the larvae are growing rapidly and the brain is continuously maturing during this life period (Wullimann & Knipps, 2000). To date, little information exists as to the extent of these changes in basal activity on toxicant responses.

There are several examples within the literature of inconsistent dose and/or behavioural responses with the same compound. For example, the brominated flame retardant tetrabromobisphenol A (TBBPA) was recently reported to reduce larval motility, from 64 nM (Noyes et al., 2015) up to 5 μM (Chen et al., 2016), whereas the plasticiser bisphenol A (BPA) has been

found to induce hyperactivity with peak activity at 100 nM (Kinch et al., 2015; Saili et al., 2012). In contrast, Jarema et al. (2015) reported no behavioural effects of TBBPA following developmental exposure at concentrations between 1.2-3.8 µM. Similarly, Wang et al. (2013) found BPA induced hypoactivity between 1-15µM, whereas Saili et al. (2012) found no behavioural effects at 1 or 10 µM. These studies all used minor alterations in methodology in comparison to one another, which included differences in larval age and the time of testing, as well as water temperature, photo-periods before and during testing, strain, toxicant purity, dosing regimen (single vs multiple), and the number of light cycles during the test, to name but a few. Of particular note, Noyes et al. (2015) and Saili et al. (2012) reared their embryos/larvae in constant darkness prior to behavioural testing (R. Tanguay, personal communication), whereas Jarema et al. (2015) and Wang et al. (2013) used a day/night (DN) cycle during rearing. This is of particular interest, as photoperiods play an important role in fish developmental biology and the development of circadian rhythms (Hurd and Cahill, 2002), but the effect on toxicity testing remains unknown.

Initially, our objective was to investigate whether larval age during day five (*i.e*. 96, 100, and 118 hpf) or the photo-regime during rearing, continuous darkness *vs.* a DN cycle, could influence the behavioural effects on larval zebrafish motility exposed to different doses of either BPA or TBBPA. Leading on from this, we explored whether a mechanism previously identified to explain hyperactivity in larval zebrafish exposed to BPA, whereby BPA acted via androgen receptors to induce aromatase B expression (Kinch et al., 2015), could explain the hypoactivity we observed when using an alternative methodology. In addition, we also assessed whether rearing larvae in constant darkness could influence the level of anxiety-like behaviour compared to larvae reared on a DN cycle, and whether the arena size could influence behavioural results in response to BPA exposure.

2. Methods

2.1 Chemicals. Stock solutions of TBBPA (97% purity, Sigma Aldrich), BPA (>99% purity, Sigma Aldrich), fluvestrant (ICI, >98% purity, Sigma Aldrich), flutamide (FLU, Sigma Aldrich), fadrozole hydrochloride (FAD, ≥98% purity, Sigma Aldrich), and 17α-ethinyl estradiol (EE2, Sigma Aldrich) were prepared in dimethyl sulfoxide (DMSO, high performance liquid chromatography grade, Sigma Aldrich). The final concentration of DMSO in all test concentrations of TBBPA, BPA, ICI, FLU, FAD, EE2, and the solvent control, was 0.01%.

2.2 Fish husbandry. The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences, Oslo, Norway. The unit 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 zebrafish were maintained at 28°C under a 14:10 light/dark photoperiod at the Norwegian University of Life Sciences (NMBU), Oslo, Norway. Adult care and breeding were conducted in accordance with the protocols under the IACUC. Fish were fed three times per day, twice with artemia and once with dry feed (Special Diet Services, UK). To generate embryos, adults were placed in spawning tanks in the afternoon, the fish were spawned following the onset of light (08:00) the next day, and the embryos collected (09:00) and maintained in embryo media (60 µg/ml Instant Ocean® sea salts) until the time of exposure.

2.3 Chemical exposure. Fertilised embryos were transferred by pipette into clear polystyrene 96-well plates (Nunc™ MicroWell™) and continuously exposed under static conditions to TBBPA, BPA, or the solvent control from 6-7 hpf until the time of testing (i.e. the larvae were tested with the

chemical present in the media). One embryo was placed into each individual well. All behavioural experiments were repeated six times.

For the initial comparison of larval age and rearing photo-regime, five nominal concentrations of BPA ranging from 1 nM to 10 μM and a solvent control were equally distributed across two 96 well plates (n=8/concentration/96 well plate. Total n=96/group in total). For TBBPA, five nominal concentrations, ranging from 150 pM to 1.5 μM, and a solvent control were equally distributed across one 96 well plate (n=16/concentration. Total n=96/group). The highest concentrations of both TBBPA and BPA were below those found to be teratogenic (< 5% of the embryos from any one group were dead and/or deformed).

For EE2, one nominal concentration of 10 nM and a solvent control were equally distributed across a 96 well plate (n=12/concentration. Total n=72/group). We selected this dose of EE2 as it induces aromatase B overexpression in larval zebrafish (Brion et al., 2012; Chung et al., 2011).

For mechanistic studies, larvae where co-exposed to BPA and either ICI (1 μM), FLU (6.18 μM), or FAD (1 μM). Here, embryos were distributed over one 96 well plate (n=8/treatment. Total n=48/group).

To assess thigmotaxis, larvae were distributed in 24 well plates and embryos were exposed from 6-7 hpf until the time of testing. Only one treatment (10 μM and 100 nM BPA for the DN cycle and constant darkness, respectively), plus the solvent control were used per plate (n=12/concentration/replicate. Total n=72/group).

When 24 well plates were used, 1 ml of exposure media was added to each well, whereas only 200 μl of media was used for each well in 96-well plates. For all exposures, embryos were reared in an incubator at 28°C. The light cycle within the incubator was 14:10 light/dark. Where complete darkness was required, the embryos were reared in the same incubator as the embryos on a DN cycle, but the well plate was wrapped in aluminium foil.

2.4 Larval behaviour. Behavioural tests were conducted using a ViewPoint® Zebrabox and the accompanying video tracking software (ViewPoint Life Sciences, Lyon, France). A difference of ≥ 5 pixels between each consecutive frame (25 frames per second) was set as the threshold for the detection of movement. Behavioural testing was undertaken at three time points for BPA, 96, 100, and 118 hpf, two time points for TBBPA, 96 and 100 hpf, and one time point for EE2, 96 hpf. These corresponded to tests beginning 90 minutes (09:00), 330 minutes (13:00), and immediately after the cessation of light in the incubator (07:30), for 96, 100, and 118 hpf, respectively. For each time point, different larvae were used to assess behaviour (i.e. no individual larvae was tested more than once). Larval behaviour, including the cumulative distance travelled and the time spent active per minute, were simultaneously measured for all larvae on a plate during a light-dark-light cycle that lasted for a total of 40 minutes and consisted of 20 minutes of light, 10 minutes of darkness, and a final 10 minutes of light. The light level was set to 100 % (7.45 Klux, TES 1337 light meter) on the ViewPoint software during the lighted periods, and 0 % (0 lux) during the dark periods when infrared light is used to track larval activity. When using 24 well plates, the arena was split into two zones, a centre zone and an outer zone, to assess thigmotaxis as detailed in Schnorr et al. (2012). Previous work has demonstrated that 5 dpf larvae treated with anxiolytic compounds are more active in the inner zone, whereas anxiogenic compounds increase the amount of movement in the outer zone (Schnorr et al., 2012; Richendrfer et al., 2012). After the behavioural test, the larvae were inspected with a stereo microscope to identify dead or deformed larvae. Deformities included spinal aberrations, yolk sac or cardiac edema, aberrations in pigmentation, and loss of equilibrium. As we only had one behavioural testing unit, it was not possible to compare the motility of fish kept on the DN cycle vs complete darkness from the same batch of embryos at the same age. Therefore, for experiments using 96 well plates different batches of embryos were used for fish kept on a DN cycle as to constant darkness, but the same breeding populations were used for both. In contrast, when using 24 well plates the same batches of embryos were used for larvae reared on a DN cycle and those reared in constant

darkness, but here those reared on a DN cycle were tested at 99 hpf (12:15) and those reared in constant darkness at 100 hpf (13:00).

With BPA, a further set of tests were used to study the effect of dark acclimation on larvae reared on a DN cycle. Here, embryos were exposed in 96 well plates to a solvent control or 0.1, 1, or 10 µM BPA across four plates (*N* = 16/concentration/plate) and maintained on a DN cycle at 28°C. Behavioural tests were carried out between 100-102 hpf (13:00-15:00). At 2, 4, 6, and 24 hrs prior to behavioural testing, one plate per time point was covered in aluminium foil to induce dark acclimation. Larval motility was then assessed during a behavioural test consisting of 20 mins of light, and 5 mins of darkness.

2.5 RNA isolation and RT-qPCR (reverse transcription-quantitative PCR). Larvae that had been exposed to a solvent control or 10 nM EE2 from 6-96 hpf, and undergone behavioural analysis at 96 hpf, were sampled for RT-qPCR. Total RNA was isolated from 11-12 larvae per biological replicate using the QIAzol lysis reagent (Qiagen, Hilden, Germany) followed by on column purification by NucleoSpin RNA kit (Macherey-Nagel) including a DNase treatment (DNase I, Amplification Grade Thermo Fisher Scientific) after RNA isolation according to the manufacturer's instructions. Three biological replicates per each treatment and control were used.

RNA concentration was measured using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, USA). All samples had an OD A260/A280 ratio of ≥ 2.0. A total of 200 ng of RNA was used for cDNA synthesis reaction using a Tetro cDNA synthesis kit (Nordic BioSite, Norway), and 5 ng was used in the qPCR reaction in triplicate per sample using Express SYBR GreenER SuperMix with premixed ROX (Invitrogen) according to the manufacturer's recommendations. Transcript levels were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the standard cycling program: 50 °C for 2 minutes, 95 °C for 2 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute, and the melting curve analyses were applied. The transcript levels of aromatase B

(AroB) and vitellogenin (vtg) were analysed with beta actin 1 and elongation factor alpha included as housekeeping genes. Primer sequences are presented in **Table S1**.

Negative controls with no added template were included for all primer pairs (no template control), and no RT control reactions for each sample and each primer pair were run in qPCR in order to check for genomic DNA contamination (no RT control). Initial analysis of the RT-qPCR data was performed using RQ Manager 1.2 (Applied Biosystems). A standard deviation of ≤ 0.3 per triplicate was accepted. The fold change was calculated by ΔΔCt method i.e. transcript levels relative to the control and normalized to the two housekeeping genes.

2.6 Statistical analysis. Data were transferred to R version 2.15.0 (R Development Core Team, http://www.r-project.org) for behavioural analyses. Individual larvae were considered as the experimental unit. Prior to statistical analysis, visual examination of the data confirmed that the dose responses to BPA and TBBPA followed similar trends between each independent experiment. Significance was assigned at $P < 0.05$. All dead and deformed larvae were discounted for behavioural analyses. For both compounds, only total motility during the dark phased was analysed as movement was minimal during the light periods. For BPA, the mean cumulative time spent moving during the initial 5 minutes of the dark period was analysed for comparisons to previous work (Saili et al., 2012; Kinch et al., 2015). For TBBPA, the mean cumulative distance moved during the entire 10 minute dark period was analysed for comparisons to previous work (Jarema et al. 2015; Noyes et al. 2015). For the thigmotaxis analysis, we also analysed the percentage of the total distance moved in the outer zone as in Schnorr et al. (2012).

Data were checked for normality following visual examination of plots (i.e. histograms and/or q-q plots). Linear mixed effect models were used for all analyses. For parametric data, the dependent variable was the cumulative time spent active for BPA or the cumulative distance travelled for TBBPA. Where data was non-parametric, these data were ranked and the rank was set as the dependent variable. Dose and larval age were included as categorical independent variables,

and replicate was included as a random effect to account for any variability between tests. In the initial models, dose and age were allowed to interact. If there was no significant interaction, then the model was simplified by removing the interaction. To assess individual doses to the controls, we used the contrast results provided within R for linear models.

3. Results

3.1 Larval age and rearing photoperiod can give contrasting results

For larvae exposed to BPA and maintained in constant darkness, there was no dose effect or interaction with larval age (**Fig. 1A**). When using a DN cycle, 10 µM BPA exposure resulted in consistent hypoactivity, but there was no interaction between dose and larval age (**Fig. 1B**).

There was a significant interaction between dose and larval age for larvae exposed to TBBPA and maintained in constant darkness. Specifically, the low doses of 1.5 and 15 nM tended to be hyperactive at 96 hpf, but hypoactive at 100 hpf whereas the high dose of 1.5 µM had no real tendency at 96 hpf, but was hypoactive at 100 hpf (**Fig. 1C**). When using a DN cycle, there was no interaction between dose and larval age, but 150 nM and 1.5 µM resulted in hypoactivity (**Fig. 1D**). Therefore, the lowest observed effect concentration was 150 nM when using a day/night cycle, compared to 1.5 nM when using constant darkness.

3.2 Dark acclimation gives contrasting results from day/night cycles

As using constant darkness vs a DN cycle led to contrasting dose responses, we wanted to understand whether a short period of dark acclimation could modify the results obtained following DN rearing. Therefore, we reared larvae on a DN cycle, but used a period of dark acclimation of between 2-24hrs prior to behavioural testing. We found that using this methodology we were unable to detect any behavioural effects of BPA exposure on zebrafish larvae (**Fig. 1E**).

3.3 Bisphenol A: testing a proposed mechanism

Previously Kinch et al. (2015) found co-exposure with the androgen receptor antagonist FLU could rescue a behavioural effect attributed to BPA exposure, but co-exposure with the estrogen receptor antagonist ICI did not. In contrast, were unable to rescue BPA induced hypoactivity with either FLU or ICI, nor the aromatase inhibitor FAD (**Fig. 1F**). Indeed, the addition of FLU led to a significant interaction effect with a greater reduction in activity compared to BPA alone. As a positive

control for aromatase B induction, we also exposed larvae to the synthetic estrogen 17α-ethinyl estradiol (EE2). However, there was no effect on behaviour in 96 hpf larvae following exposure to 10 nM EE2 even though the transcript levels of the molecular markers for estrogenic exposure vtg and AroB were upregulated compared to controls (**Figure S1**).

3.4 Methodology influences the behaviour of the controls

We had determined that the photo-regime experienced during larval rearing could influence our behavioural outcomes in larvae treated with BPA and TBBPA, but it was unclear why. However, it was clear that the photo-regime prior to behavioural testing had pronounced effects on the behaviour. For example, the total time spent active was significantly greater in those larvae reared on a DN cycle compared to those in constant darkness irrespective of larval age. In addition, larvae reared on a DN cycle and given a 2-24 hr period of dark acclimation were characterised by an intermediate level of activity (**Fig. 2A**). Larval age was also found to influence behaviour as we found consistent increases in the distance moved with age in larvae reared in constant darkness, but no such trend in larvae reared on a DN cycle (**Fig 2A**). Furthermore, a large percentage of those larvae maintained in constant darkness remained quite static in that they failed to move more than one body length (> 4 mm) during the dark period of the behavioural test, but this effect decreased with age (25, 12, and 5% at 96, 100, and 118hpf, respectively). In contrast, in larvae reared on a DN cycle < 1% of the population were static during the dark period at any given age. In addition, larvae reared on a DN cycle showed peak activity at minute 21, which is immediately after the onset of darkness during the behavioural test (**Fig. 2B**), whereas those larvae reared in constant darkness showed peak activity at minutes 23-26, several minutes after the onset of darkness (**Fig. 2C**). Finally, when considering the lighted period of the behavioural test, irrespective of the rearing photo-regime, larvae at 118 hpf began to show low levels of movement compared to the freeze behaviour shown at 96 and 100 hpf (**Fig. 2BC**).

3.5 Thigmotaxis and arena size

As general activity is typically associated with the level of anxiety (Kalueff et al., 2013), we asked whether keeping larvae in constant darkness *vs.* DN cycle could influence any other endpoint of anxiety. Therefore, we assessed the degree of thigmotaxis, or wall hugging, in five-day-old larvae. Consistent with our previous findings, larvae reared in constant darkness spent significantly less time moving than those larvae maintained on a DN cycle also when using 24 well plates (**Fig. 3A**). However, there was no effect of rearing photo-regime on the level of thigmotaxis (**Fig. 3B**). Unexpectedly, in DN reared larvae, 10 µM BPA exposure in a 24 well plate resulted in hyperactivity (**Fig. 3A**) in contrast to our previous finding of hypoactivity when using 96 well plates (see **Fig. 1B**). Furthermore, BPA treated larvae showed significantly greater levels of thigmotaxis (**Fig. 3B**). When using constant darkness, 100 nM BPA had no effect on activity levels or thigmotaxis (**Fig. 3B**).

4. Discussion

We found methodology to have a significant influence on the outcome of a larval zebrafish assay when assessing behavioural responses to two known endocrine disruptors. Of particular importance to toxicity testing, we found both larval age and the photo-regime resulted in significant changes in the minimum toxic effect concentration for both compounds tested. In addition, the same concentration of a given compound could increase, decrease, or have no effect on motility in larvae of the same age depending on methodology. These results demonstrate the importance of accurately recording and standardising methodology when conducting behavioural tests. In addition, these results highlight the challenges of translating behavioural data from larval zebrafish to other vertebrate models and their potential role in the risk assessment of new and existing compounds.

A major objective for toxicity testing is to determine lowest effect concentrations. We found this value was dependent on methodology. Taking TBBPA as an example, the lowest effect concentrations ranged from 1.5 nM to 150 nM. Furthermore, both compounds had age- and photo-regime specific effects. Such differences have serious implications on the translation of large-scale screening studies that rely on only one time point and/or photo-regime (Jarema et al., 2015; Noyes et al., 2015). For example, we have demonstrated that minor alterations in methodology determined whether or not BPA tested positive or not for behavioural effects.

Notable differences in locomotion were found between non-treated DN and constant darkness reared larvae. We could not associate these behavioural effects with any gross differences in morphological staging between the two photo-regimes. Previously, Kazimi and Cahill (1999) also reported that dark rearing had no gross effect on morphological staging. However, DN cycles are essential for establishing behavioural rhythmicity in zebrafish larvae from as early as 2 dpf (Hurd and Cahill, 2002) and light exposure from 2 dpf is required in order to establish the rhythmic expression of clock genes (Hurd and Cahill, 2002), cell cycles (Dekens, 2003) and melatonin levels (Kazimi and Cahill, 1999) in zebrafish. Rearing in constant darkness is also known to influence visual behaviour

(Bilotta, 2000) and long-term survival. For example, Villamizar et al. (2014) found that rearing larvae under constant darkness up until 5 dpf, when they were transferred to a DN cycle, resulted in a significant increase in mortality compared to controls at 30 dpf. Finally, it is unclear what effect the photo regime itself may have on the physicochemical properties of the test compound within the media, for example its rate of degradation. Therefore, further work is required in order to understand whether these factors may be influencing toxicity testing.

Of particular interest is how our alterations in methodology led to significant differences in behavioural outcomes. For example, BPA induced hypo- or hyper-activity, or had no effect at all dependent on methodology. Of particular note, we are unable to explain why 10 µM BPA leads to hyperactivity following testing in a 24 well plate, but hypoactivity when using a 96 well plate. However, well size can influence the degree of basal activity, with larvae maintained in the larger wells of a 24 well plate moving more than when maintained in a 96 well plate (Padilla et al., 2011). We also provide evidence that BPA induced motility effects may have several modes of action. That is, whereas androgen receptors and aromatase B overexpression appear to be associated with the mechanism behind BPA induced hyperactivity in larvae reared in constant darkness (Kinch et al., 2015), we found no support for this pathway in BPA induced hypoactivity in DN reared larvae. Similarly, we found the potent aromatase B inducer 17α-ethinyl estradiol had no effect on larval behaviour. However, in support of Kinch et al. (2015), we were also unable to rescue the BPA induced behavioural response with an estrogen receptor antagonist.

We found larval age interacted with the dose of TBBPA when rearing larvae in constant darkness. Here, we note that zebrafish larvae reared on day/night cycles begin to display circadian rhythms in locomotor activity at 4 days post fertilisation (Hurd and Cahill, 2002). Therefore, it is possible that time of day may explain the larval age and dose interaction, as larvae of different ages were tested at different times of the day. However, Hurd and Cahill (2002) reported that the earliest development stage at which environmental light signals were able to set the phase of weak

behavioural rhythms was related to the time of the differentiation of the pineal, around 24 hpf. Therefore, as we exposed our fertilised embryos to constant darkness from 6 hpf, it appears unlikely the age effect in larvae exposed to TBBPA and constant darkness is influenced by the time of day of testing.

As larval zebrafish are not classified as live animals until the onset of feeding, they are viewed as an alternative to live animal testing, including within developmental neurotoxicity assessment (Coecke et al., 2007). Numerous behavioural endpoints are available with this model, but how does one interpret an "effect" seen within zebrafish larvae to human neurotoxicity? Here, it is generally agreed that as long as the molecular basis between the two biological systems are similar, as it is between zebrafish and humans, then if one sees an "effect" in zebrafish it is likely a similar effect will occur in humans (Coecke et al., 2007). However, we highlight that the behavioural outcomes in zebrafish larvae can be contrasting or non-existent depending on methodology. A lack of standardisation and validation has been identified as a barrier to the broader acceptance of small fish models within toxicology (Planchart et al., 2016). Therefore, there is a need to determine how alterations in methodology alter basal behaviour in order to gain more insight into the mechanistic pathways and relevance of potential neurotoxins.

In conclusion, we found a dramatic effect of methodological practices on behavioural outcomes, not only following exposure to toxic compounds, but also in basal activity levels. Notably, the same concentration of a compound could lead to hyperactivity, hypoactivity, or have no behavioural effect, depending on methodology. As such, inconsistencies within the literature may reflect a lack of standardisation in methodological practices. We believe our results exemplify a need for greater transparency in all methodological practices. In addition, we recommend further validation of this test system in order to understand how differences in methodology influence basal activity as this may improve our ability to translate larval zebrafish behaviour into other animal models and thereby refine risk assessment.

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Figure legends

Figure 1. **Locomotor activity in larval zebrafish exposed to bisphenol A (BPA) and tetrabromobisphenol A (TBBPA).** Dose responses were influenced by larval age and/or rearing photo-regime. In (E), all larvae were originally reared on a DN cycle, but were given a period of dark acclimation from 2-24 hrs prior to behavioural testing at 100-102 hours post fertilisation (hpf). In (F), larvae were reared on a DN cycle and co-exposed to 10 µM BPA and either 6.18 µM flutamide (FLU), 1 µM fadrozole (FAD), or 1 µM fulvestrant (ICI) before behavioural testing at 98 hpf. In (A-E), an asterisk indicates an overall significant effect (LME, P <0.05) of dose compared to the control, whereas subscript letters indicate a significant interaction between dose and larval age. In (F), all BPA larvae showed significantly less activity compared to controls (not indicated on the graph for clarity), and the subscript letter indicates a significant interaction effect between the control (in this case FLU) and co-exposure with BPA. Values are means ± SE. For (A-E), n 90-96 for all groups, for (F), n = 46-48 for all groups.

Figure 2. **A comparison of the controls between different methodologies and larval ages**. (A) The time spent active during the initial 5 minutes of the dark period of the behavioural test. Larvae were reared on either a day/night cycle (DN) or), under constant darkness (CD), or reared on a DN cycle with a period of dark acclimation of between 2-24 hrs (DA). Different lower case letters indicate a significant group effect (LME, P < 0.05). Larvae were tested at 96, 100, or 118 hours post fertilisation (hpf). Values are means ± SE, n = > 90 group. The distance moved in the controls during the behavioural test in larvae previously reared on (B) a day/night cycle or (C) in constant darkness and tested at different hpf. Values are means ± SE, n = 92-190 $minute^{-1}$ age^{-1}.

Figure 3. Locomotor activity and thigmotaxis in larval zebrafish reared in 24 well plates. (A) Time spent active and (B) thigmotaxis in larvae reared on a DN cycle (DN) and exposed to 10 µM bisphenol A (BPA), or reared under constant darkness (CD) and exposed to 100 nM BPA. Asterisk represents a

significant effect of either the rearing photo-regime or BPA (LME, $P < 0.05$). Values are means ± SE, n = 70-72 for all groups.

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