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Getting more out of the zebrafish light dark transition test

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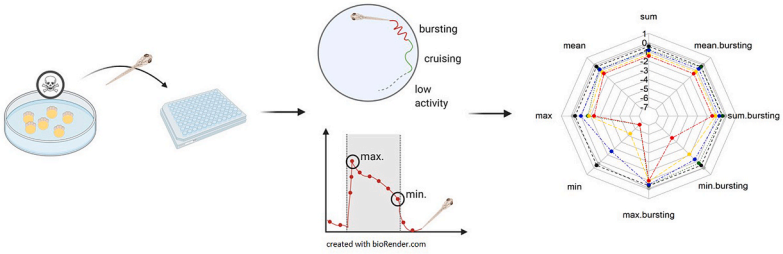
HIGHLIGHTS

- Integration of multiple parameters can aid mechanistic understanding
- Approach enabled distinction between different substance classes
- Better characterisation of behavioural response during light period
- Radar charts improve LDT data representation and interpretation

GRAPHICAL ABSTRACT



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ABSTRACT

In (eco-)toxicological studies the light/dark transition (LDT) test is one of the most frequently used behaviour assays with zebrafish eleutheroembryos. However, study results vary regarding data presentation and analysis and mostly focus on a limited amount of the recorded data. In this study, we investigated whether monitoring two behavioural outcomes (time and distance moved) together with analysing multiple parameters can improve test sensitivity and data interpretation. As a proof of principle 5-day old zebrafish (*Danio rerio*) eleutheroembryos exposed to either endocrine disruptors (EDs) or acetylcholine esterase (AChE) inhibitors were investigated. We analysed conventional parameters such as *mean* and *sum* and implemented additional endpoints such as *minimum* or *maximum* distance moved and new parameters assessing the bursting response of eleutheroembryos. Furthermore, changes in eleutheroembryonic behaviour during the moment of the light to dark transition were added. To improve data presentation control-normalised results were displayed in radar charts, enabling the simultaneous presentation of different parameters in relation to each other. This enabled us to identify parameters most relevant to a certain behavioural response. A cut off threshold using control data was applied to identify parameters that were altered in a biological relevant manner. Our approach was able to detect effects on different parameters that remained undetected when analysis was done using conventional bar graphs on - in most cases analysed - averaged, mean distance moved values. By combining the radar charts with additional parameters and by using control-based thresholds, we were able to increase the test sensitivity and promote a deeper understanding of the behaviour response of zebrafish eleutheroembryos in the LDT test and thereby increased its usability for behavioural toxicity studies.

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1. Introduction

Zebrafish *(Danio rerio)* embryos have become a popular tool to investigate neuroactive/-toxic effects and over the last decade, the number of behavioural studies using zebrafish increased rapidly (De Esch et al., 2012; Irons et al., 2010; Kalueff et al., 2016; Leuthold et al., 2019; Ogungbemi et al., 2019; Zindler et al., 2020). Based on the increasing body of literature, the light/dark transition (LDT) test with 5 to 7-day old eleutheroembryos/larvae may be considered the most applied assay for determining early life stage behaviour (Dach et al., 2019; Irons et al., 2010; Jarema et al., 2015; Velki et al., 2017). The principle of this test is to assess the behavioural response of free swimming, eleutheroembryonic zebrafish towards a visual stimulus (switch from light to dark and vice versa), tracking changes in activity with an automated video tracking system (Irons et al., 2010; MacPhail et al., 2009). In the assay, normal behaviour is characterised by low basal activity in light and a substantial increase of activity upon the switch to darkness (Irons et al., 2010; MacPhail et al., 2009). Using the LDT, perturbations of this normal response were found following exposure to ethanol or cocaine, substances also known to influence mammal behaviour (Crozatier et al., 2003; De Wit et al., 2000; Feola et al., 2000).

Although this assay is frequently applied in (eco)toxicology, there is an ongoing discussion about how the data should be analysed. Usually, a simplistic analysis is done using one value per endpoint such as the mean or median distance or time moved over a defined period, e.g., the dark period (Jarema et al., 2015; Velki et al., 2017; Yen et al., 2011). Thereby temporal information about the behavioural response over time is lost (Ingebretson and Masino, 2013) as activity peaks and lows are smoothened. More advanced analysis methods are available, such as the general linear mixed models (GLM) or the use of benchmark concentrations (Hsieh et al., 2019; Liu et al., 2017). The application of those models requires a decent statistical background as well as informatics skills. Due to these preconditions, such models are difficult to apply. Besides, those approaches also focus on only one parameter at a time like distance, though taking the temporal changes into consideration (Hsieh et al., 2019; Liu et al., 2017).

In this study, we investigated whether the interpretation of LDT behavioural data can be improved by measuring two instead of one outcome (time and distance moved) and by adding multiple parameters. Furthermore, a cut-off threshold using control data was applied to identify parameters that were altered in a biological relevant manner. Our aim was to increase the LDT's capability to detect effects rapidly, to identify parameters relevant to the overall behavioural response and eventually to achieve a better understanding of the behavioural response.

In a proof concept approach, acetylcholinesterase (AChE) inhibitors were used for these investigations and compared against endocrine disrupting compounds. AChE inhibiting insecticides, like organophosphates or carbamates, inhibit the cleavage of the neurotransmitter acetylcholine (ACh) by AChE, resulting in its accumulation in the synaptic cleft and a continuous activation of muscarinic and nicotinic ACh receptors. The overexcitation of postsynaptic neurons leads to seizures and death in target organisms like insects. In non-target organisms, prolonged exposure can initiate a cascade of downstream events, leading to e.g., increased oxidative stress, neuroinflammation and cell death (Beal, 1995; Ecobichon, 2001; Eisenkraft et al., 2013; Faria et al., 2015; Gao et al., 2019; Küster and Altenburger, 2007; Peña-Llopis et al., 2003; Shih and McDonough, 1997). These effects may adversely reflect on behaviour or perturb metabolic or developmental processes (Binukumar et al., 2010a,b; Bui-Nguyen et al., 2015; Velki et al., 2017; Yozzo et al., 2013; Zindler et al., 2019a,b). Endocrine disruptors (EDs) exert adverse effects via multiple pathways and mechanisms. For this study tris(1, 3-dichloro-2-propyl)phosphate (TDCPP), bis(2-ethylhexyl)phthalate (DEHP) its metabolite mono (2-ethylhexyl)phthalate (MEHP) and 4-nonylphenol (4-NP) were selected. TDCPP, an organophosphorus flame retardant, interferes with the endocrine system by perturbating steroidogenesis and causing a sex and thyroid hormone imbalance (reviewed by Wang et al., 2020). Similar effects were reported for DEHP and MEHP, while for 4-NP mainly oestrogen receptor interactions were documented (reviewed by Jie et al., 2013; Matthiessen et al., 2018; Rowdhwal and Chen, 2018; Yang et al., 2015). Due to research implying adverse effects of these substances on neurological functions and development as well as the close interaction between the endocrine and the nervous system, the named EDs were included in the behaviour investigations (reviewed by Matthiessen et al., 2018; Rowdhwal and Chen, 2018; Wang et al., 2020).

2. Material & methods

2.1. Chemicals

With exception of diazoxon (Toronto Research Chemicals inc., Canada) all chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and had at least 95% purity: Diazinon, aldicarb, paraoxon-ethyl, dichlorvos, paraoxon-methyl, DEHP, MEHP, TDCPP, 4-NP, DMSO. Stock solutions of all substances were prepared by dilution in 100% DMSO. The tested substance concentrations and CAS numbers are listed in Table S1.

2.2. Egg retrieval and embryonic exposure

Adult wild type zebrafish (AB wild type from Ruinemans, The Netherlands) were kept at 26 ± 1 °C and a 14:10 light/dark rhythm. Female and male fish were separated the evening before the experiment. The next morning mating was initiated by bringing fish back together. Fertilised eggs were transferred into zebrafish embryo test (zfet) medium (100 mg/L $NaHCO_3$, 20 mg/L $KHCO_3$, 180 mg/L $MgSO_4$, 200 mg/L $CaCl_2$) and their quality was assessed using a stereomicroscope (Motic Microscopy).

Within 2 h post fertilisation (hpf) 12 embryos were transferred into single wells of a 6-well plate and exposed towards the test substances in four concentrations at and below the NOEC for malformations. Exposure solutions were prepared by diluting the stock solutions in zfet medium shortly before exposure was initiated. The final solvent concentration in the experiments was 0.01% (v/v) DMSO. Subsequently, exposed embryos were kept in the incubator (26 ± 1 °C, 14:10 light dark rhythm) until 5 dpf when the LDT test was conducted. After 1- and 5-days post fertilisation (dpf) (eleuthero-)embryos were visually assessed for death and sublethal effects and excluded from behaviour experiments, if necessary. Exposure solutions were not refreshed during the experiment.

2.3. LDT testing

At 5 dpf, in the morning, single eleutheroembryos were transferred into the wells of a 96-well plate, in 250 µL exposure medium. The experiments were conducted in the afternoon (starting at 2 p.m.), since eleutheroembryonic behaviour is more stable between noon and afternoon (Kristofco et al., 2016; MacPhail et al., 2009) and to give eleutheroembryos time to adjust to the new environment. In each concentration/control 12 eleutheroembryos were used. The LDT assay was conducted in two alternating cycles of light and darkness, each cycle being 10 min long, starting with a light cycle. Tracking was done using a ZebraBox and the corresponding software (ZebraLab® v3, Viewpoint, France). Light intensity during light periods was 222 lux, measured using an iPhone 5 and the LuxMeterPro application (AM Power-Software). During the experiment the temperature was kept at 26 ± 1 °C, using a water bath. All substances were tested in independent quadruplicates or at least triplicates and every replicate included a negative control (NC = zfet medium) and solvent control (SC = 0.01% DMSO). The following analyses were conducted solemnly on 5 dpf eleutheroembryos. For the sake of simplicity, we will further refer to this life-stage as embryo.

2.4. Data evaluation

Only data of the second light/dark period of the LDT assay was used for data analysis and data presentation. The first period was used as an acclimation phase to reduce the variability in the embryonic response, analogous to (Leuthold et al., 2019).

2.4.1. Bar graph analysis

Data on the total distance moved by embryos was averaged over the respective 10 min dark/light period. Subsequently, the Shapiro-Wilk test was applied to assess the data distribution. If data were normally distributed statistically significant differences compared to the SC were assessed using a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. Non-normal distributed data were analysed using the non-parametric Kruskal-Wallis and a Dunn's multiple comparison post-hoc test. This approach was chosen due to its frequent use in other research articles (Steele et al., 2018; Velki et al., 2017; Zindler et al., 2019a,b).

2.4.2. Data processing

Behaviour data processing was done using Microsoft Excel 2016. Behaviour was assessed as distance moved and time moved, categorised as <0.3 cm/s = low activity, 0.3–1 cm/s = cruising and >1 cm/s = bursting. Total distance is calculated for 1-min time bins. Data are either summed to obtain *sum* or averaged over the 10 min duration of light (or dark) period to obtain *mean*. (Table 1). The same was performed for bursting events to obtain respectively *sum.bursting* and *mean.bursting* (Fig. 1, red line). The total distance moved was calculated from low activity, cruising and bursting data, while the total time moved was calculated from cruising and bursting data (Fig. 1). .

To retrieve minima and maxima of total distance moved each embryo was screened for the shortest (*min.*) and longest (*max.*) distance swam within 10 min and based on 1-min. time bin data (Fig. 1 B). Thus, 12 data points were obtained per parameter (*min./max.*), making a total of 24 (12 x *min*, 12 x *max*) values, which were then averaged to form either *min.* or *max.* (Table 1) distance moved. The same was done to retrieve minima and maxima for total time moved and for bursting parameters, with the latter only considering bursting data (*min./max. bursting*, Table 1).

All calculations were done for both, the 10-min light and the 10-min dark period.

Behavioural investigations regarding the substances influence on the transition from light to dark were done by calculating the difference of the total distance moved/time moved during the last minute in light and the first minute in darkness. For this analysis absolute values were used. Data analysis was performed as described in 2.4.1.

2.4.3. Radar charts

To promote symmetry within the data, processed behaviour data (2.4.2) was normalised to its respective SC and $\log_2$ transformed. Replicate data was averaged. Table 1 presents the parameters used for the radar charts.

The radar charts were created in RStudio (RStudio Team, 2019) using R version 4.0.2 (R Core Team, 2020) and the fmsb package version 0.7.0 (Nakazawa, 2019).

Table 1
Overview of radar chart parameters depicting the parameters name, its function and the respective unit.

parameter	Explanation	unit
sum	sum total distance/time moved	[cm/10 min and s/10 min]
mean	mean total distance/time moved	[cm/min. and s/min.]
sum.bursting	sum distance/time moved	[cm/10 min and s/10 min]
mean. bursting	mean distance/time moved	[cm/min. and s/min.]
max	mean of maximum total distance/time moved	[cm/min. and s/min.]
min	mean of minimal total distance/time moved	[cm/min. and s/min.]
max.bursting	mean of maximal distance/time moved	[cm/min. and s/min.]
min.bursting	mean of minimal distance/time moved	[cm/min. and s/min.]

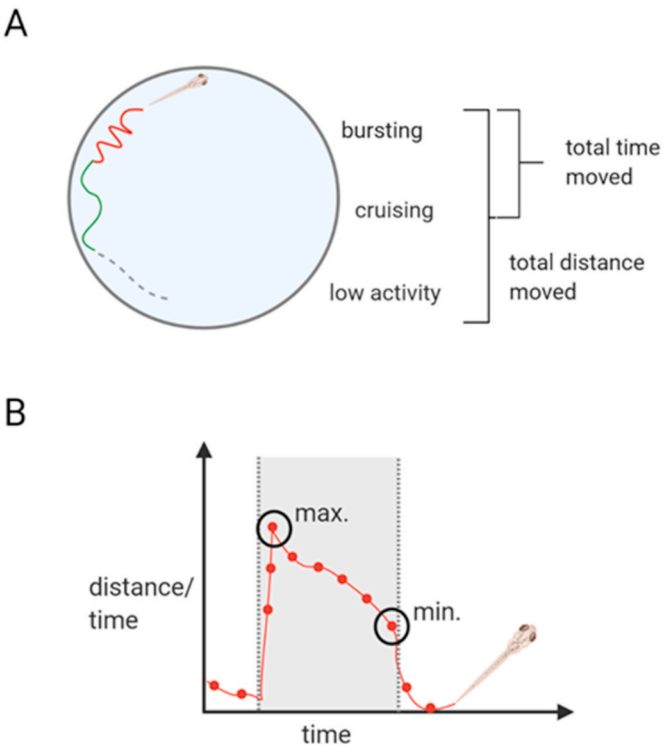

Fig. 1. LDT experiment parameter introduction The figure displays how data were retrieved from one exemplary embryo. A) Total distance was calculated based on zebrafish embryo low activity (grey dashed line, <0.3 cm/s), cruising (green line, 0.3–1 cm/s) and bursting (red line, > 1 cm/s). Total time moved was calculated based on cruising and bursting only as including low activity would add up to the whole minute of tracking. Bursting (red line) was used as an additional parameter for which *sum* and *mean* were calculated. B) depicts an exemplary behaviour course of an embryo in darkness (grey background) and the timely occurrence of *min.* and *max.*. Dots represent exemplary 1-min time bins, which were extracted for *min* and *max* analyses. Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4.4. Control based cut-off thresholds

To identify biological relevant effects on behaviour after exposure, cut-off thresholds based on control data were calculated. Cut-off's were set at the 95th percentile based on the combined NCs and SCs data for each parameter (Table 1). To analyse the variation in controls (SCs/NCs), data of all SC's or NCs were averaged to form an overall mean either called TSC (total SC) or TNC (total NC). Subsequently, NC and SC data from single exposure experiments were normalized to either TSC or TNC and again $\log_2$ transformed (Fig. 2 and S1 & S2).

For the light period this resulted in a ±0.73-fold change cut-off for distance and time moved. Dark period cut-offs were determined as fold changes of ±0.40 for distance moved and ±0.52 for time moved (Figure S3). The defined cut-offs were applied to all exposure experiments. Data ranging within the 95th percentile limit were considered normal and hence depicted the no effect range. Data above or below the cut-off was considered a biologically relevant behavioural change and subjected to rudimentary statistical analysis.

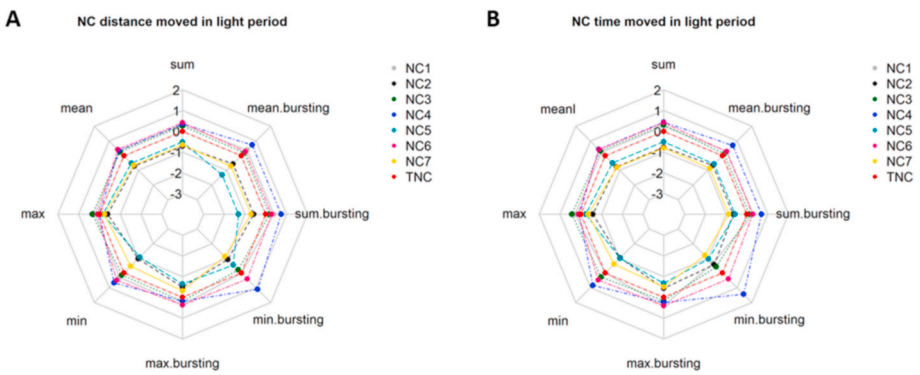

Fig. 2. Behavioural response of 5 dpf embryos during the light period. Plotted data depict respective controls normalized to the mean of all controls (NTC/STC) from the same condition (distance/duration) and log2 transformed. A) shows the distance moved by NCs and B) the time moved during the light period. NC = negative control (zfet), TSC = total NC.

2.4.5. Radar chart statistical evaluation

Statistical analysis was done on un-averaged $\log_2$ transformed replicate values. To enable a fast assessment of behaviour radar charts were first analysed using control-based cut-off values (see 3.1) and only endpoints that were varying from controls were further analysed for significance.

Subsequently, $\log_2$ transformed data were analysed by first checking for multicollinearity. Second, a one-way ANOVA followed by a Dunnett's multiple comparison test were applied to identify significant differences between treatments and controls. Additionally, a multivariate analysis of variance (MANOVA) was run to assess if a multivariate dependence between the treatment and the different behaviour parameters could be established. All analysis on radar chart parameters was done using RStudio (R version 4.0.2, R Core Team, 2020; RStudio Team, 2019) and the multcomp package (Bretz et al., 2011; Hothorn et al., 2020).

Due to the $\log_2$ transformation, data of SC were set to zero which is why statistical analysis had to be run against the NC. Also because of this, the comparison of SCs and NCs against the TSC/TNC had to be done using absolute values. Furthermore, data analysis for parameters *min. bursting* and *min.* was complicated in light periods as normal embryonic behaviour involves a reduced locomotor activity during light periods which results in several zero values for minimal activity parameters. This fact makes the calculation of a $\log_2$ fold change impossible. Therefore, these parameters were analysed using their absolute values, too.

3. Results & discussion

Zebrafish embryos were exposed to ten different substances from two different compound classes and a LDT test was performed at 5 dpf. Afterwards, a standard data analysis and an extended data analysis was conducted to test if it was possible to increase test sensitivity and data interpretation.

3.1. Monitoring one outcome vs. two outcomes

To assess if measuring two outcomes during the test would improve test sensitivity and biological interpretation the time and distance moved were recorded and analysed. The data in Tables 2 and 3 show large similarities between both outcomes, but also differences. For example, during darkness some substances appeared to cause a decreased mean distance moved, while the mean time moved was unaffected (see e.g. paraoxon-ethyl exposed embryos during darkness, Tables 2 and 3). Likewise, exposure to some substances, mainly EDs, increased the time embryos moved but not the distance (see e.g. DEHP exposed embryos during darkness, Tables 2 and 3). These differences between distance and time moved were mainly observed in dark and to a lesser extent in light periods. Furthermore, parameter variability of both outcomes was dependent on the respective illumination condition (light/dark) and comparable between outcomes of the same condition (Figure S 4). Accordingly, it may be assumed, that utilising distance and time moved in dark periods can help detect the differential influence of test substances on these outcomes, possibly hinting towards the mechanistic background. By only using one outcome (distance or time), these differences could have been missed completely, which emphasises the need to include both. Biological interpretation with only one parameter and two outcomes is difficult but still could result in hints for the underlying mechanisms. There are various reasons for these findings and in order to contribute to their elucidation the below-mentioned parameters were included in the investigation.

3.2. Adding multiple parameters

The benefits of investigating multiple parameters to achieve a detailed description of behaviour have been formulated years back (Ingebretson and Masino, 2013). In literature, different approaches to address this issue can be found (Altenhofen et al., 2019; Gauthier and Vijayan, 2018; Steele et al., 2018; Wang et al., 2018), showing the value and necessity of depicting and analysing multiple parameters (Ingebretson and Masino, 2013). Here, radar plots were used to display multiple parameters in relation to each other thus improving data interpretation (Figures S 1–2, S5-7). Radar plots have already been used by Steele et al. (2018), where the authors depicted data on bursting, cruising and freezing normalised to the control. In comparison, we normalised the data and performed a $\log_2$ data transformation to improve symmetry in the data and to depict parameters of varying range next to each other, thus promoting the comparability of parameters. Furthermore, parameters that we considered biologically relevant, such as *minima* and *maxima* during bursting were added to the graphs. The parameters were selected, because they indicate the embryos' ability to respond to an external stimulus. A response with relevance to survival in an ecological context.

Statistical analysis of complex behavioural data is very challenging and there is an ongoing discussion on which statistical test is best suited and how to cope with the multiple testing problem. We, therefore, decided to apply only a simple, rudimentary statistical testing but focus on the identification of biologically relevant changes using cut-off thresholds from control data (2.4.4). Basically, if the value of a parameter for a treatment was exceeding the control data variation over all experiments, it was considered non-normal and therefore biologically relevant. Data considered biologically relevant was then investigated using statistical measures to confirm their statistical relevance. Data that passed the cut-off but was not significantly different from controls (by means of statistics, 2.4.5) were considered a trend.

Table 2
Summary of radar chart analysis using the distance moved and according to pre-evaluations via the cut-off criteria. If data met the cut-off statistical analysis was performed. Arrows indicate the direction of effect with ↑ indicating an increased and a ↓ decreased activity. The occurrence of ↑/↓ was due to the test setup using two different plates and thus two different controls. Asterisks indicate statistically significant results. *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$. Arrows without indication of significance implicate behavioural trends, based on cut-offs. T = treatment, the numbers 1-4 represent the four test concentrations, with 1 being the lowest and 4 the highest concentration.

substance	parameter																													
light period	min				max				mean				sum				min.bursting				max.burstinng				mean.bursting				sum.bursting			
T	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
aldicarb																	↓	↓	↑	↑												
diazinon				↑							↑				↑					↑												
diazoxon	↑	↑	↑	↑													↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
dichlorvos	↓	↓	↓	↓	↓*		↓	*↓	↓*	↓	*↓	***↓	↓	↓	*↓	***↓	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
paraoxon-ethyl																		↓	↓													
paraoxon-methyl				↓																↓				↓								↓
4-NP				↓																↓				↓								↓
DEHP	↑	↑	↑	↑					↑	↑		↑	↑	↑		↑		↑		↑	↑			↑	↑	↑		↑	↑	↑		↑
MEHP																	*↓										↑				↑	
TDCPP	↑								↑				↑				↑	↑	↑	↓	↑				↑	↑			↑	↑		
dark period	min				max				mean				sum				min.bursting				max.burstinng				mean.bursting				sum.bursting			
aldicarb																↓																
diazinon																			↓								↓				↓	
diazoxon																			↑													
dichlorvos	*↓	**↓	***↓	***↓			**↓	**↓	*↓	*↓	**↓	***↓	*↓	*↓	**↓	***↓			↓				↓*				*↓	*↓			*↓	*↓
paraoxon-ethyl																	↓				↓				↓				↓			
paraoxon-methyl	↓		↓	↓														↓	↓	↓		↓	↓	**↓				*↓				*↓
4-NP																																
DEHP			↑	↑													↑	↑		↑					↑			↑	↑			↑
MEHP			↑														↓						↓		↓		↑		↓		↑	
TDCPP																																

Table 3
Summary of radar chart analysis using the time moved and according to pre-evaluations via the cut off criteria. If data met the cut off statistical analysis was performed. Arrows indicate the direction of effect with ↑ indicating an increased and a ↓ decreased activity. The occurrence of ↑/↓ was due to the test setup using two different plates and thus two different controls. Asterisks indicate statistically significant results. *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$. Arrows without indication of significance implicate behavioural trends, based on cut-offs. T = treatment, the numbers 1-4 represent the four test concentrations, with 1 being the lowest and 4 the highest concentration.

substance	parameter																													
light period	min				max				mean				sum				min.bursting				max.burstinng				mean.bursting				sum.bursting			
T	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
aldicarb																	↓	↓														
diazinon											↑				↑					↓												
diazoxon	↑	↑	↑														↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
dichlorvos	↓	↓	↓	↓	↓*		↓	↓*			↓	↓			↓	↓		↓	↓	↓							↓	↓			↓	↓
paraoxon-ethyl																	↓															
paraoxon-methyl		↓		↓						↓		↓		↓		↓		↓		↓				↓		↓		↓		↓		↓
4-NP		↓																	↓				↓				↓			↓		
DEHP	↑	↑	↑	↑					↑	↑		↑	↑	↑		↑		↑		↑	↑	↑		↑	↑	↑		↑	↑	↑		↑
MEHP					↓												↓															
TDCPP	↑								↑				↑				↑			↓	↑				↑	↑			↑	↑		
dark period	**min**				**max**				**mean**				**sum**				**min.bursting**				**max.burstinng**				**mean.bursting**				**sum.bursting**			
aldicarb																	↓	↓			↓	↓			↓	↓			↓	↓		
diazinon											↓				↓				↓				↓									
diazoxon																																
dichlorvos	*↓	***↓	***↓	***↓			***↓	***↓			↓*	↓**			↓*	↓**			↓	*↓	***↓				***↓		**↓	***↓			**↓	***↓
paraoxon-ethyl																																
paraoxon-methyl			↓	↓														↓	↓	↓		↓		**↓			↓	*↓			↓	*↓
4-NP																																
DEHP				↑								↑				↑		↑		↑					↑	↑	↑	↑	↑	↑	↑	↑
MEHP	↑	↑	↑																													
TDCPP							↑	↑																			↑				↑	

3.2.1. Maximum and minima

Maximum and minimum parameters were included to improve the biological interpretation of measured outcomes. Of all analysed parameters, *min. bursting* was the most sensitive, passing the cut-off threshold in 17 out of 20 assessments for distance moved and 15 out of 20 for time moved (Table 2). It was also the most sensitive parameter for each light phase (10 light, 7 dark) and considering the exposure concentration, providing the lowest NOEC. Therefore including *min. bursting* as parameter can increase LDT sensitivity. Yet, it must be taken into consideration that minima parameters can include zero values (no movement) which make statistical analysis problematic. This is especially the case during light periods where zebrafish embryos show a very low and sometimes no activity. Due to this, minima parameters of the light period belonged to the most variable parameters in this approach (Figure S4). In comparison, variability of maxima parameters was generally low, particularly for *max* (Figure S4), but they were passing the cut-off less frequently (*max.bursting* in 11/10 and *max* in one/two out of 20 assessments for distance/time moved, Table 2). To summarise, the use of maxima parameters during light periods, may be favoured over minima parameters, though both can be used to explore dark period data.

Considering the timely occurrence of minima and maxima during each light or dark period, data did not show definite, substance class specific differences. Though, during dark periods AChE inhibitors and EDs tended to distribute to different areas of the heatmap. The upper part of the heatmap largely contained insecticidal substances, which could be hinting towards a different impact (Figure S10). Also, during the 10 min dark period, maxima values tended to occur earlier, compared to minima. A trend that is in line with literature, reporting strongly increased activity at the beginning of dark periods which slowly declines over time (Irons et al., 2010; MacPhail et al., 2009; Padilla et al., 2011). During light periods substance class specific separation was less clear and maxima and minima appeared to distribute randomly throughout the 10 min observation period (Figure S11).

3.2.2. Bursting

Furthermore, we focused on bursting behaviour as parameter, as it is suspected to be a good indicator for the embryonic escape or light-seeking response, with the latter resulting in large angle movements upon the switch to darkness (Buick and O'Malley, 2000; Burgess and Granato, 2007). Behavioural parameters measuring bursting were already successfully used to identify substance effects on fish embryo behaviour (Drummond and Russom, 1990; Steele et al., 2018). Furthermore, bursting parameters may be used for investigating and identifying e.g., zebrafish embryos with insufficient muscle development or function (Naganawa and Hirata, 2011). *Mean.bursting* and *sum. bursting* parameters for e.g., the distance moved passed the cut-off in 12 out of 20 behaviour assessments. Although these parameters were sufficiently sensitive, it is advised to use them for dark period analyses as they vary during light periods (Figure S4), which might result from the high variation of *min.bursting* in this period.

As mentioned before effects on muscle development could impact the bursting capability of embryos (Naganawa and Hirata, 2011). Our own findings support this assumption, as reduced *max.bursting* was seen in zebrafish embryos exposed to dichlorvos and paraoxon-methyl, known AChE inhibitors (Tables 2 and 3, Fig. 5). A lack of AChE has been shown to disrupt muscle development in embryonic zebrafish (Behra et al., 2002; Brennan et al., 2005; Yozzo et al., 2013). This example demonstrates the usefulness of *max.bursitng,* also due to its low variability in the dark, which is why this parameter is recommended for future investigations on behaviour (Figure S4).

3.2.3. Transition event

In addition, a parameter representing the transition from light to dark was included to achieve an advanced representation of the direct response to the stimulus. The transition point from light to dark was utilised by Thomas et al. (2019) as a sensitive parameter to identify non-normal behaviour in exposed embryos. In the present work, it was found that exposure to 25 and 30 μM dichlorvos was sufficient to significantly reduce the time embryos moved during the transition, compared to the SC (Fig. 3 B). Nevertheless, these embryos covered a distance comparable to the SC (Fig. 3 A). This might indicate an impaired response to the light transition. Dichlorvos did not induce morphological changes in the retina at a concentration of 12.8 μM (Stengel et al., 2018). But the impact of higher concentrations is unknown, hence an effect on the retina structure cannot be excluded. Furthermore, dichlorvos may impact signal transmission or translation into movement (Binukumar and Gill, 2010c; Klein et al., 2019). Regarding the other substances, no clear impact on the transition was noted but a tendency may be indicated for 4-NP and paraoxon-methyl (Figure S 9).

3.3. Standard vs multiple parameters

The standard analysis was performed using only the mean of the total distance moved. This analysis represents one of the most common ways used to analyse LDT data. By utilising this method, a significant effect on behaviour was detected only for dichlorvos in the dark period and at higher concentrations (Fig. 4, Figures S9 & 10). Similarly former research, using this standard method, mostly detected a decreased activity during dark periods (Cao et al., 2018; Jin et al., 2015; Kristofco et al., 2016; Velki et al., 2017). The NOEC for dichlorvos was 22.5 μM. The other tested substances did not result in any significant effects (Figure S13 & 14). In Table 2 an overview of effects and trends, found by adding multiple parameters, is depicted. A total of eight out of 10 compounds showed an impact/influence on at least one of the parameters, six affected multiple parameters, during darkness. In light periods, nine out of 10 substances influenced multiple parameters. Yet, significant effects were observed for dichlorvos, paraoxon-methyl and MEHP, only. AChE inhibiting compounds, with exception of diazoxon, were found to or tended to reduce embryonic activity while EDs tended to increase it. The latter was mainly observed during light periods. The cause for these tendencies is unclear as the impact of EDs on neuronal development is not fully understood (summarised by Lupu et al., 2020). However, endocrine systems are of major importance for accurate neuronal development (Bakker, 2019; Bayless and Shah, 2016; Dach et al., 2017; Denley et al., 2018; McCarthy, 2008; Remaud et al., 2017). Exposure to TDCPP, DEHP, MEHP and 4-NP has been linked to endocrine dysregulations e.g. of the steroid or thyroid hormone system, reduced neurite outgrowth and post-synaptic function as well as altered behaviour (Dishaw et al., 2014 a,b; Fraser et al., 2017; Jie et al., 2013; Matthiessen et al., 2018; Rowdhwal and Chen, 2018; Wang et al., 2020; Wang et al., 2017; Yang et al., 2015). Consequently, changed locomotor behaviour in the LDT test could be due to an interference of EDs with neuronal development in the embryos. However, the results obtained in this work are trends and hence further research is necessary to confirm or dismiss them. Reduced locomotion upon exposure to AChE inhibitors was reported multiple times and is presumably due to cholinergic overstimulation and subsequent secondary neurotoxic mechanisms, of which some will be discussed below by means of an example (Faria et al., 2015; Peña-Llopis et al., 2003; Schmitt et al., 2019; Watson et al., 2014).

As only dichlorvos induced an effect in the standard approach we will focus our exemplary comparison of data interpretation and biological understanding on this substance. In the standard approach the distance embryos moved was decreasing in the dark phase in a dose dependent manner (Fig. 4). This could be caused by reduced energy, impaired muscle function, less sensitivity to the stimulus (Behra et al., 2002; Bui-Nguyen et al., 2015; Scott and Sloman, 2004), basically anything that accounts for less movement. With this method it is not possible to gain any further information about the behavioural response and a biological interpretation is therefore very limited.

When monitoring two outcomes and multiple parameters more

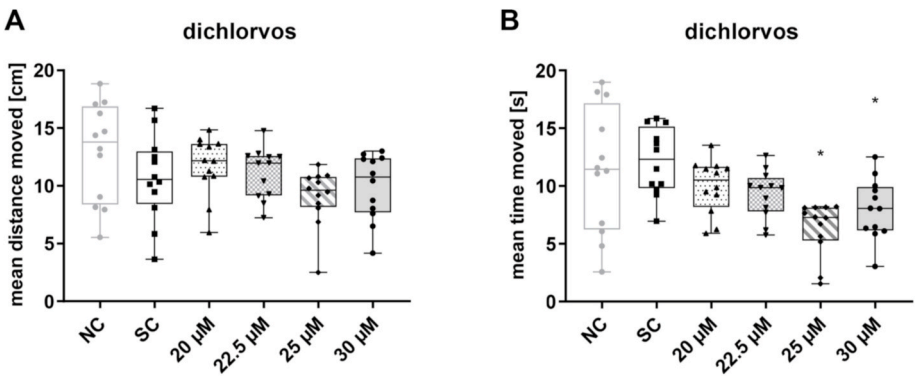

Fig. 3. Results representing the transition from light to darkness. The differences due to the transition are expressed as mean distance moved [cm] (A) or mean time moved [s] (B) calculated from the last minute during light and the first minute of the dark period. Error bars indicate the standard deviation. Data were derived from three replicates with 12 organisms each. * = $p < 0.05$.

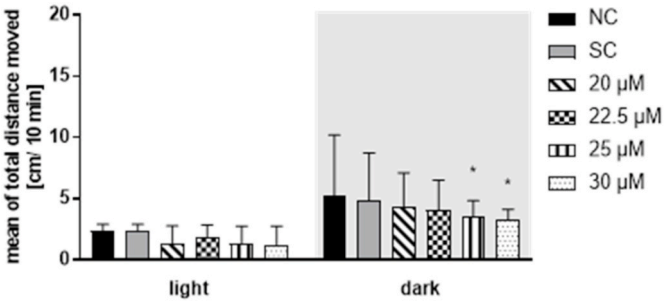

Fig. 4. Bar graphs from the LDT test using 5 dpf embryos and exposed for five days to dichlorvos. Data are displayed as mean of the total distance moved/10 min time interval. Error bars indicate the standard deviation. Data were derived from three replicates with 12 organisms each. SC = 0.01% DMSO, n = 3, * = $p < 0.05$.

information can be retrieved. The radar charts revealed a relevant impact of dichlorvos on the distance and time embryos swam, with minima and bursting parameters showing the most pronounced alteration (Fig. 5, Tables 2 and 3). This effect was not limited to the dark period, as indicated by the standard evaluation, but also occurred in the light phase. Detecting this effect was probably possible because of the $\log_2$ transformation of the data, which pronounced the differences between treatments and controls. Furthermore, higher concentrations of dichlorvos significantly ($p \leq 0.05$) affected the transition response (Fig. 3). Combined results of the transition endpoint (3.2) and the radar chart analysis suggested an activity increase of the embryos upon the switch to darkness. However, the increased activity seemed to cease fast as indicated by the reduced distance and time embryos moved, as well as the low minima and maxima values in the radar charts (Fig. 5). Embryos appeared to respond strongly to the dark stimulus but compared to controls, were unable to keep up their activity. Taken together, these findings could indicate exhaustion. Support for this hypothesis may be related to dichlorvos ability to cause oxidative stress and produce reactive oxygen species (ROS) (Bui-Nguyen et al., 2015; reviewed by Das, 2013; Kanthasamy et al., 2008; Smith et al., 2000). Oxidative stress was found to contribute to an impaired ATP production in mitochondria of rat liver (Binukumar et al., 2010a,b) and adversely affecting the liver energy metabolism in adult zebrafish (Bui-Nguyen et al., 2015). Furthermore, Bui-Nguyen et al. (2015) noted degenerative changes in skeletal muscles. These findings were in line with Behra and colleagues' data, showing that AChE activity was necessary for developing and maintaining the axial muscle apparatus (Behra et al., 2002). Hence, the observed swimming impairments could be a result of a lack of energy and neuro-muscular perturbations. These assumptions are well in line with recent literature demonstrating comparable effects in zebrafish after exposure to chlorpyrifos-oxon (Gómez-Canela et al., 2017). A direct impact on behaviour, due to dichlorvos' ability to impede AChE function appeared unlikely as the substance hydrolyses quickly in aqueous media and did not affect ACh concentrations after five days of exposure (Binukumar et al., 2010a,b; Fent, 2013; Tufi et al., 2016).

The presented results yield valuable information and our approach may be used in future work to indicate mechanisms by which, substances other than dichlorvos might influence behaviour on the physiological level. Kokel et al. (2010) also found a link between the predicted toxic mechanism and the behavioural response by using the early developmental photomotor response test, yet they were unable to entirely clarify the mechanisms underlying the observed behaviour phenotype. Therefore, including multiple parameters in behaviour assessments and analysis is highly recommended.

Due to the limited number of test substances it was not possible to determine, which of the endpoints was most sensitive for detecting dose-

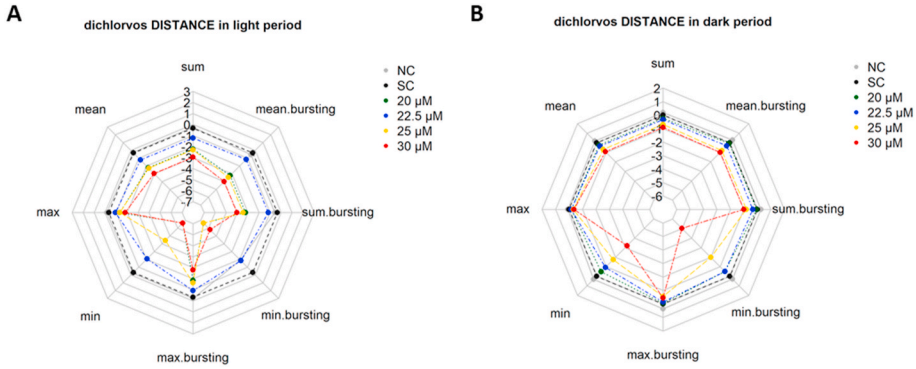

Fig. 5. Results from the LDT test using 5 dpf embryos exposed for five days to dichlorvos. Radar charts depict behavioural parameters regarding the distance moved by embryos in light (A) and darkness (B). Sum/mean total = sum/mean total distance moved by embryos, max./min. total = mean of maximum/minimum total distance moved by embryos, max./min. bursting = mean of maximal/minimal distance moved by embryos during burst, sum/mean bursting = sum/mean total distance moved by embryos during burst. Maximum/minimum refers to the farthest/shortest distance measured for embryonic movement. SC = 0.01% DMSO, n = 3.

dependent effects. However, results on dichlorvos (Figure S6 & 7) indicated that 1) the illumination condition might be relevant for detecting a dose-response relationship and 2) that multiple of the presented parameters can be utilised. Though, it is noteworthy to keep in mind that not all substances exert monotonic activity alterations, as has been documented for D-amphetamine and ethanol which caused a biphasic behaviour response in zebrafish embryos (Irons et al., 2010). In the future, experiments on additional substances and substance classes will help in refining this matter.

3.4. Biological relance vs statistical significance

In numerous scientific publications the detection of statistically significant outcomes was documented and has quite often been treated equally to a biologically relevant effect. However, biological relevance and statistical significance are not necessarily linked (EFSA, 2011). Therefore, the European Food Safety Authority (EFSA) advised considering statistical significance derived by statistical analysis in the context of biological relevance (EFSA, 2011).

With regards to this, the present study focused on the detection of biologically relevant effects that were, subjected to simple statistical analysis, to enable a fast and easy application. Yet, we acknowledge that using mean values or a linear statistical model such as ANOVA does not reflect the complexity of the LDT behavioural response (Liu et al., 2015; Zhang et al., 2017a, 2017b). To facilitate statistical data interpretation, multivariate testing strategies and beforehand power analysis could be included.

4. Conclusion

The aim of this work was to promote the understanding of LDT behaviour data by evaluating additional parameters thus extracting biologically relevant information about the response.

This was achieved by analysing two behavioural outcomes (distance and time moved) and adding multiple parameters which improved LDT test sensitivity and data interpretation. In doing so, we found that a substance can affect various aspects of the behavioural response. Moreover, $\log_2$ transformation of data facilitated the detection of effects during the light period. This enabled us to show, that EDs tended to affect locomotion mostly during light periods, while AChE inhibitors tended to exert an impact on dark period behaviour. However, it was not possible to identify individual substances based on the parameters they affected. In general, there is potential to improve statistical analysis with regards to implementing multivariate statistics, especially because radar charts themselves cannot depict variation in the data. Still, using radar charts proved to be suitable for displaying different behaviour parameters together in a comprehensive manner thus providing an overview of parameters influencing behaviour in the LDT test. Another advantage in using radar charts is the ability to easily integrate new parameters, e.g., turn angle or velocity. Considering the single parameters, their suitability to describe behaviour was found to depend on the illumination condition. Bursting and maxima parameters may be used in the analysis of both, light and dark, while the use of minima parameters should be restricted to dark periods. However, the parameter of choice largely depends on the research question and should be selected accordingly. This might also include using minima parameters for light period analysis, e.g., in the case of hyperactive behaviour.

Compared to conventional analysis methods additional and more detailed information was obtained from the same experiments and displayed simultaneously. The resulting insights can be used as indicators for the identification of new research directions contributing to the elucidation of a substances' toxic mechanism. Future research could utilise our approach to identify new targets that may be the subject of subsequent molecular analyses.

Author's contributions

Ann-Cathrin Haigis: Conceptualisation, investigation, visualisation, writing- original draft. Richard Ottermanns: Formal analysis, writing- review & editing. Andreas Schiwy: Writing- review & editing, supervision. Henner Hollert: Writing- review & editing, supervision. Jessica Legradi: Conceptualisation, writing- review & editing, supervision.

Declaration of competing interest

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

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Appendix A. Supplementary data

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